REMARKS

Reconsideration of this application is respectfully requested.

Claims 454-575 are pending in this application. No claim has been amended or deleted. No new claims have been added. Accordingly, the claims 454-575 are presented for further examination on the merits.

The First Rejection Under 35 U.S.C. §112, First Paragraph

Claims 459-472 and 474-575 stand rejected under 35 U.S.C. §112, first paragraph. In the Office Action (pages 2-3) the Examiner stated that these claims contain:

> "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention for reasons of record. This is a "new matter" rejection.
>
> Support was not found where indicated in the specification, nor elsewhere, for the following limitations in Claims 459-472 and 474-575:
>
> Claims 459-463, specific chemical compositions of linkages;
>
> Claims 464-472, 482-569, specific identity of labels and points of attachment of the "SIG" moiety to internal phosphates;
>
> Claims 474-477 and 570-575, the "composition" limitation, in addition to the above identified limitations.
>
> Applicant argues in the response, pages 30-32, that support was found in various portions of the specification. This argument is not persuasive. These portions merely recite support for covalent attachment of a SIG moiety to a phosphate moiety and a SIG moiety to the oxygen on a phosphate moiety. Note, there is no support for the attachment of the SIG moiety to the phosphate atom of the phosphate moiety, only to the oxygen atom."

This rejection is respectfully traversed. Example V on page 57 of the specification does in fact disclose Sig attached directly to the phosphorous atom of the phosphate moiety (PM). Additionally an enlarged copy of the Halloran Figure 1, including reaction 2, does reveal that Halloran depicts the Phosphate moiety

attachment to a phosphate atom. Examiner acknowledges the Halloran disclosure at pages 3 and 4, and one only needs to glance at the Halloran reference to see the attachment points to understand the invention being disclosed and conveyed by this application.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of this rejection under 35 USC 112.

The Second Rejection Under 35 U.S.C. §112, First Paragraph

Claims 454-575 stand rejected for enablement under 35 U.S.C. 112, first paragraph, as "containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, for reasons of record." (Office Action, page 3).

The Examiner states on pages 3-4 of the Office Action that:

> Applicant argues that the prior art, such as Halloran, supports enablement. This argument is not persuasive for several reasons:
>
> Halloran discloses the attachment of a specific signal moiety, a protein, to the phosphorus atom of the phosphate moiety using a specific linker, a -C-$(CH_2)_4$-N- chain. In contrast, the claims are drawn to a much broader category, a generic "SIG" moiety and linkage, and specific compounds such as those recited in claim 464 -- magnetic, hormone, metal containing "SIG" moieties, for example. Thus, the scope of the enablement is not commensurate with the scope of the claims.
>
> In addition, several of the reference articles are drawn to labeling a mononucleotide and express doubt about labeling an oligonucleotide. Armstrong et al., Eur. J. Biochem. 0:33-38, 1976, teaches that the labeled mononucleotides are "strong competitive inhibitors" of the reaction which is necessary to produce a labeled oligonucleotide from a labeled mononucleotide. See Armstrong, p. 33, col. 1. This reaction is the use of the labeled mononucleotide as a substrate for the polymerase mediated synthesis of the oligonucleotide.
>
> While Armstrong teaches that some labeled mononucleotide will be incorporated, Armstrong teaches no guidance as to which of the myriad labels within the scope of these claims will function in the claimed invention. Lacking any guidance in the specification and in view of the breadth of the claims, it would require undue experimentation in order to enable a reasonable number of embodiments of these claims. The skilled artisan would have to

experiment with various SIG moieties, linkages and attachment sites, as well as various reaction protocols and protecting groups.

This enablement rejection is respectfully traversed. One skilled in the art and armed with the present disclosure would have sufficient guidance to practice the invention over the entire claimed range. Applicants are in the process of gathering expert testimony and articles to support their position and will submit such information in a supplemental response.

Applicants respectfully request reconsideration and withdrawal of the rejection for lack of enablement.

The First Rejection Under 35 U.S.C. §103

Claims 454-575 stand rejected under 35 U.S.C. § 103 for being unpatentable over Gohlke et al., US Patent 4,378,458, 3/1983, filed 3/1981 (Gohlke) in view of Sodja et al., Nucleic Acids Res., 5(2):385-401, 1978 (Sodja) and further in view of applicant's admissions.

On pages 4-5 of the Office Action, the Examiner stated that:

> Applicant argues that the opening of the ring sugar in Sodja distinguishes Sodja from the e current application. It is argued that the terminal nucleotide, with the open sugar, is outside the scope of the claims.
>
> This argument is not persuasive. Sodja reads on the claimed invention because of the scope of the term "SIG" moiety. There is nothing in the limitation "SIG" which would exclude the terminal nucleotide, with the open ribose sugar from being a part of the "SIG" moiety. The "terminal" nucleotide in the claimed product would be the second nucleotide from the end in the Sodja reference, which has a closed ribose sugar ring.
>
> Applicant argues that Gohlke does not teach labeling ribonucleotides and thus does not suggest the claimed DNA products. This argument is not persuasive. First, many of the claims of this case are not limited to DNA products but read on ribonucleotides. Second, it is Gohlke in view of Sodja which is the basis of the rejection. There is no evidence that Gohlke cannot be applied to Sodja for the expected benefit of generating other types of labeled oligonucleotides using the Gohlke labels.

This obviousness rejection is respectfully traversed. There is no teaching or suggestion of how to modify Sodja to open-up DNA or otherwise attach to a phosphate of a DNA. Gohlke does not cure the defects of Sodja, at best it would label a RNA disrupted ring.

The Second Rejection Under 35 U.S.C. §103

Claims 454-575 stand rejected under 35 U.S.C. § 103 as being unpatentable over Halloran et al., J. Immunol. 96(3):373-378, 1966 or Miller et al. 20(7):1874-1880, 1981.

On pages 5-6 of the Office Action, the Examiner stated that:

> Both Halloran and Miller teach specific labels, (SIG moieties such as proteins and thiophosphates) attached to nucleic acids. See Halloran p. 373, Fig. 1 and col. 2; Miller p. 1874, col. 1. These prior art references differ from the claims in the recitation of some specific labels and linkages. It would have been *prima facie* obvious, however, to one of ordinary skill in the art at the time the invention was made to substitute any linker or label in the methods of Halloran and Miller for the expected benefit of constructing multiple labels. Given the fact that diverse labels such as proteins and thiophosphates, the ordinary artisan would have reasonably expected any moiety used as a label to function in the claimed invention.
>
> Repeating an important point from a previous Office action, applicants arguments to the 35 U.S.C. § 112, first paragraph rejections have provided strong evidence of obviousness and vice-versa. For example, the references used to support enablement, Halloran and Miller add evidence of obviousness.
>
> It is important that the arguments for patentability explain, that the prior art supplied by applicant, for example Halloran and Miller, can buttress the specification--providing needed evidence that the thin "SIG Phosphate" disclosure both "describes" and "enables" the detailed invention now claimed--but that these same prior art references do not render the claims obvious.
>
> Furthermore, the criticisms of the obviousness rejections must be made without undermining the enablement rejection. For example, if arguing that Halloran and Miller are somehow "non-enabled" one must justify how the specification can be enabled. After all, the prior art contains much more detail than that found in the specification.
>
> Finally, the specification is held to a higher standard than the teachings of the prior art supplied in an obviousness rejection. As stated in a previous office action:

This obviousness rejection is respectfully traversed. Applicants respectfully maintain that the differences between the instant invention and the combination of cited documents would not have been obvious to one of ordinary skill in the art at the time of the invention. Halloran was published 29 years before this application was deposited, Miller was published 14 years before; it was the applicants who first came to the realization of this invention. Thus, it would be unpermitted hindsight for one to use the current application to apply teachings from the prior art to "reinvent" the invention.

Reconsideration and withdrawal of the obviousness rejection is respectfully requested. Applicants are in the process of detailing additional arguments to support their position of non-obviousness over the cited references and will submit these arguments in a supplemental response shortly.

In view of the above amendments and foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the anticipation rejection, thereby placing each of claims in allowable condition.

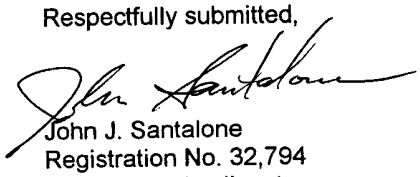
****

SUMMARY AND CONCLUSIONS

No other fees are believed due in connection with this filing. In the event that any other fee or fees are due, however, the Patent and Trademark Office is authorized to charge the amount of any such fee(s) to Deposit Account No. 05-1135, and to credit any overpayment thereto.

In view of the above discussion of the issues, Applicants respectfully submit that all of the instant claims are in allowable condition. Should it be deemed helpful or necessary, the Examiner is respectfully invited to telephone the undersigned at (212) 583-0100 to discuss the subject application.

Respectfully submitted,



John J. Santalone
Registration No. 32,794
Attorney for Applicant

ENZO DIAGNOSTICS, INC.
c/o Enzo Biochem, Inc.
527 Madison Avenue, 9th Floor
New York, New York 10017
Tel.: (212) 583-0100
Fax.: (212) 583-0150

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(71) Applicant: ENZO BIOCHEM, INC.
325 Hudson Street
New York, N.Y. 10013(US)

(72) Inventor: ENGELHARDT, Dean
173 Riverside Drive
New York, N.Y. 10024(US)
Inventor: RABBANI, Elazar
69 Fifth Avenue
Nwe York, N.Y. 10003(US)
Inventor: KLINE, Stanley
235 Lincoln Place
Brooklyn, N.Y. 11217(US)
Inventor: STAVRIANOPOULOS, Jannis G.
515 West 59 Street
New York, N.Y. 10019(US)
Inventor: KIRTIKAR, Dollie
42-72 80 Street
Elmhurst, N.Y. 11373(US)

(74) Representative: Vossius & Partner
Siebertstrasse 4 P.O. Box 86 07 67
D-8000 München 86(DE)

(54) Modified labeled nucleotides and polynucleotides and methods of preparing, utilizing and detecting same.

(57) Nucleotides and polynucleotides, including DNA, are chemically modified or labeled so as to be capable of ready detection when attached to and/or incorporated in nucleic acid material.

EP 0 285 058 A2

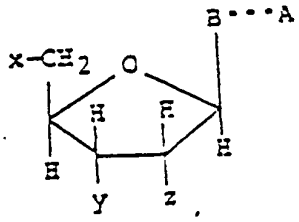
MODIFIED LABELED NUCLEOTIDES AND POLYNUCLEOTIDES AND METHODS OF PREPARING, UTILIZING AND DETECTING SAME

BACKGROUND OF THE INVENTION

It is known to produce nucleotides or polynucleotides which are radioactively labeled, such as with isotopes or hydrogen (^{3}H), phosphorus (^{32}P), carbon (^{14}C) or iodine (^{125}I). Such radioactively labeled compounds are useful to detect, monitor, localize and isolate nucleic acids and other molecules of scientific or clinical interest. Unfortunately, however, the use of radioactively labeled materials presents hazards due to radiation. Also due to the relatively short half life of the radioactive materials employed to label such compounds or materials, the resulting labeled compounds or materials have a corresponding relatively short shelf life.

It has been proposed to chemically label compounds of interest, such as nucleotides and polynucleotides, so as to overcome or avoid the hazards and difficulties associated with such compounds or materials when radioactively labeled. In the article by P.R. Langer, A. A. Waldrop and D. C. Ward entitled "Enzymatic Synthesis of Biotin-Labeled Polynucleotides: Novel Nucleic Acid Affinity Probes", in Proc. Natl. Acad. Sci., USA, Vol. 78, No. 11, pp. 6633-6637, November, 1981, there are described analogs of dUTP and UTP that contain a biotin molecule bound to the C-5 position of the pyrimidine ring through an alkylamine linker arm. The biotin-labeled nucleotides are efficient substrates for a variety of DNA and RNA polymerases in vitro. Polynucleotides containing low levels of biotin substitution (50 molecules or fewer per kilobase) have denaturation, reassociation and hybridization characteristics similar to those of unsubstituted controls. Biotin-labeled polynucleotides, both single and double stranded, are selectively and quantatively retained on avidin-Sepharose, even after extensive washing with 8M urea, 6M guanidine hydrochloride or 99% formamide. In addition, biotin-labeled nucleotides can be selectively immunoprecipitated in the presence of antibiotin antibody and Staphylococcus aurea, Protein A. These unique features of biotin-labeled polynucleotides suggest that they are useful affinity probes for the detection and isolation of specific DNA and RNA sequences.

The disclosures of this article are herein incorporated and made part of this disclosure. The subject matter of said article is comprised in EP-A2-0063879 in which additionally it is disclosed that compounds having the structure:

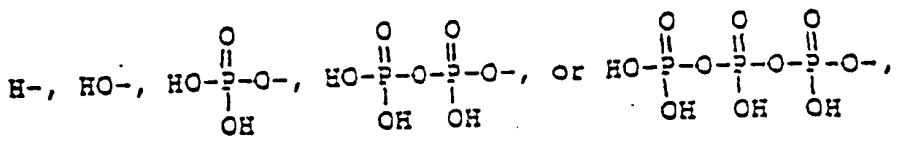


wherein B represents a purine, deazapurine, or pyrimidine moiety covalently bonded to the $C^{1'}$-position of the sugar moiety, provided that when B is purine or 7-deazapurine, it is attached at the N^{9}-position of the purine or deazapurine, and when B is pyrimidine, it is attached at the N^{1}-position:

wherein A represents a moiety consisting of at least three carbon atoms which is capable of forming a detectable complex with a polypeptide when the compound is incorporated into a double-stranded ribonucleic acid, deoxyribonucleic acid duplex, or DNA-RNA hybrid:

wherein the dotted line represents a chemical linkage joining B and A, provided that if B is purine the linkage is attached to the 8-position of the purine, if B is 7-deazapurine, the linkage is attached to the 7-position of the deazapurine, and if B is pyrimidine, the linkage is attached to the 5-position of the pyrimidine; and

wherein each of x, y and z represents



either directly, or when incorporated into oligo-and polynucleotides, provide probes which are widely usely.

Applications disclosed in EP-A2-0 063 879 include detection and localization of polynucleotide sequences in chromosomes, fixed cells, tissue sections and cell extracts. Specific applications include chromosomal karyotyping, clinical diagnosis of nucleic acid-containing etiological agents, e.g. bacteria, viruses, or fungi, and diagnosis of genetic disorders.

The disclosures of EP-A2-0 063 879 are herein incorporated and made part of this disclosure.

By way of additional background with respect to the utilization of the biotin-polynucleotides of the above-identified Langer et al article in Proc. Natl. Acad. Sci. USA, the publication by P. R. Langer-Safer, M. Levine and D. C. Ward in Genetics entitled "An Immunlogical Method for Mapping Genes on Drosophila Polytene Chromosomes", describes a method employing biotinated nucleotides as a probe for the localization of DNA sequences hybridized in situ to Drosophila polytene chromosomes. In this application these probes are detected using affinity purified rabbit antibiotin antibody as the primary antibody and fluorescenated goat antirabbit antibody as the secondary antibody. The disclosures of this Langer-Safer et al publication in Genetics are also incorporated and made part of this disclosure.

Other techniques employing biotin-labeled reagents with avidin or enzyme-labeled avidin reagents are known for the detection and determination of ligands in a liquid medium, see U.S. Patent 4,228,237. Also, it is known to effect gene enrichment based on avidin-biotin interaction, particularly as applied to Drosophila ribosomal RNA genes, see the J. Manning, M. Pellegrini and N. Davidson publication in Biochemistry, Vol. 16, No. 7, pages 1364-1369 (1977). Other publications of background interest with respect to the practices of this invention are the D. J. Eckermann and R. H. Symons article entitled "Sequence at the Site of Attachment of an Affinity-Label Derivative of Puromycin on 23-S Ribosomal RNA of Escherichia coli Ribosomes", J. Biochem, 82, 225-234 (1978); the article by S. B. Zimmerman, S. R. Kornberg and A. Kornberg entitled "Glucosylation of Deoxyribonucleic Acid-II -Glucosyl Transferases from T2-and T6-Infected Escherichia coli in The Journal of Biological Chemistry, Vol. 237, No. 2, February 1962, and the article by J. Josse and A. Kornberg "III. α-and β-Glucosyl Transferases from T4-Infected Escherichia coli", also appearing in The Journal of Biological Chemistry, Vol. 237, No. 6, June 1962.

Of further interest in connection with the practices of this invention are the publications appearing in the J. Biol. Chem., Vol. 236, No. 5, May 1961, pages 1487-1493; the same publication, Vol. 237, No. 4, pages 1251-1259 (1962); the same publication Vol. 239, No. 9, pages 2957-2963 (1964). Of special interest is the article appearing The Journal of Histochemistry and Cytochemistry, Vol. 27, No. 8, pages 1131-1139 (1979) and in the publication Nucleic Acids Research, Vol. 5, No. 9, 1977, pages 2961-2973. Also of interest is the article appearing in the publication Biochimica et Biophysica Acta by A. De Waard entitled "Specificity Difference Between the Hydroxymethylcytosine β-Glucosyl-Transferases Induced by Bacteriophages T2, T4 and T6", pages 286-304, and also the article by T. W. North and C. K. Mathews entitled "T4 Phage-Coded Deoxycytidylate Hydroxymethylase: Purification and Studies in Intermolecular Interactions", published by Academic Press, 1977, pages 898-904 and the article by E. A. Bayer and M. Wilchek entitled "The Use of Avidin-Biotin Complex as a Tool in Molecular Biology in Methods of Biochemical Analysis, Vol. 26, pages 1-45 (1980).

Other techniques useful in the practices of this invention include nick translation of DNA employing DNA polymerase. A technique for effecting nick translation is disclosed in the article by P. W. Rigby, M. Dieckmann, C. Rhodes and P. Berg entitled "Labeling Deoxyribonucleic Acid to High Specific Activity in vitro by Nick Translation with DNA Polymerase" in J. Mol. Biol. (1977), 113, 237-251. With respect to the recovery of streptavidin, such as from a culture broth of Streptomyces avidinii, the article by K. Hofmann, S. W. Wood, C. C. Brinton, J. A. Montibeller and F. M. Finn entitled "Iminobiotin Affinity Columns and their Application to Retrieval of Streptavidin" in Proc. Natl. Acad. Sci. USA, Vol. 77, No. 8, pp. 4666-4668 (1980), discloses a suitable approach for the recovery of streptavidin from a strepavidin-containing material, such as from a culture broth. Streptavidin is useful as a reagent in one of the practices of this invention.

The aforementioned publications are herein incorporated and made part of this disclosure.

SUMMARY OF THE INVENTION

In accordance with the practices of this invention nucleotides are modified, such as at the 5 position of pyrimidine or the 7 position of purine, preparatory for the preparation therefrom of nucleotide probes suitable for attachment to or incorporation into DNA or other nucleic acid material. In the practices of this invention nucleotides, i.e. nucleic acids, preferably are modified in a non-disruptive manner such that the resulting modified nucleotides are capable of incorporation into nucleic acids and once incorporated in nucleic acids the modified nucleotides do not significantly interfere with the formation or stabilization of the double helix formed of the resulting nucleic acids containing the modified nucleotides. The non-disruptive modification of nucleotides and nucleic acids incorporating such modified nucleotides is in contrast with those modifications of nucleotides which are characterized as a disruptive modification in the sense that the resulting disruptively modified nucleotides and nucleic acids containing the same block proper double helix formation. In the practices of this invention, the nucleotides are desirably modified at the 5 position of the pyrimidine or the 7 position of the purine. The nucleotides so modified are non-disruptively modified and nucleic acids containing such nucleotides are capable of forming a double helix arrangement.

Broadly, in another aspect of the practices of this invention various methods are useful for the tagging or labeling of DNA in a non-disruptive manner. For example, biotin is added on the end of a DNA or RNA molecule. The addition of biotin is accomplished by addition of a ribonucleotide. The 3',4' vicinal hydroxyl groups are oxidized by periodate oxidation and then reduced by a borohydride in the presence of biotin hydrazide. Alternatively, carbodiimide can also be used to couple biotin to the aldehyde group.

Another technique for tagging nucleic acid material such as DNA or RNA involves the addition of a large marker to the end of a DNA or RNA molecule. One example of this technique is the addition of a molecule, e.g. lysylglycine, where the amino groups are tagged with biotin. Another example would be to follow the procedure set forth hereinabove but employing carbodiimide as the cross-linking agent. Still another example of this technique would be to produce a biotinylated dA:dV double helical polymer and to ligate this polymer to the probe prepared in accordance with this invention.

Another technique for tagging DNA in a non-disruptive manner involves the isolation of dPyrTP having a putricine or spermidine on the 5 position from PS16 or phage-infected cells. If desired, dPyrTP is made from phage DNA and phosphorylated to dPyrTP followed by modification of the polyamine side chain by means of standard nucleophilic reagent NHS-biotin.

Another technique for tagging DNA in a non-disruptive manner involves the addition of glucose to 5-hydroxymethylcytosine (5 HMC) in DNA using T4 phage glycoslyating enzymes followed by screening by means of a lectin-based assay.

Still another method for tagging DNA in a non-disruptive manner involves 5-HMC-triphosphate made from the hydrolysis of T4-DNA followed by phosphorylation of the 5HMCMP to 5 HMCTP. 5HMCTP is then incorporated into DNA using polymerase I. Thus, any DNA can be modified to have non-disruptively incorporated therein 5 HMC.

A method for tagging DNA in a mildly disruptive manner involves reacting nucleic acids in the double helical form with alkylating reagents as for example benz(o)pyrene diol epoxide or aflatoxin. Under appropriate conditions the N^2 group of guanine, the N^4 group of adenosine or the N^4 group of cytosine are alkylated. These modified nucleotides can be directly detected with antibodies or can be used as linking arms for the addition of a reporter molecule such as biotin.

Preferred embodiments of the present invention are explained in detail in the following enumeration.

1. A nucleotide having the general formula P-S-B-Sig wherein P is the phosphoric acid moiety, S the sugar or monosaccharide moiety, B being the base moiety, the phosphoric acid moiety being attached at the 3' and or the 5' position of the sugar moiety when said nucleotide is a deoxyribonucleotide and at the 2', 3' and or 5' position when said nucleotide is a ribonucleotide, said base being a purine or a pyrimidine, said base being attached from the N1 position or the N9 position to the 1' position of the sugar moiety when said base is a pyrimidine or a purine, respectively, and wherein said Sig is a chemical moiety covalently attached to the base B of said nucleotide, said Sig when attached to said base B being capable of signalling itself or makes itself self-detecting or its presence known.

2. A nucleotide in accordance with item 1 wherein said nucleotide is a deoxyribonucleotide.

3. A nucleotide in accordance with item 1 wherein said nucleotide is a ribonucleotide.

4. A nucleotide in accordance with item 1 wherein said chemical moiety Sig is chemically attached to B at the N7 position when B is a 7-deazapurine, at the C5 position when B is a pyrimidine and at the C8 position when B is a purine.

5. A nucleotide in accordance with item 1 wherein Sig is attached to B at a position such that an oligonucleotide or polynucleotide containing said nucleotide is capable of forming a double-stranded ribonucleic acid, a double-stranded deoxyribonucleic acid or a DNA-RNA hybrid, or when said nucleotide is incorporated into said oligonucleotide or polynucleotide.

6. A nucleotide in accordance with item 1 wherein Sig is attached to B at a position such that said nucleotide is capable of being incorporated into or to form a double-stranded ribonucleic acid, a double-stranded deoxyribonucleic acid or a double-stranded deoxyribonucleic acid-ribonucleic acid hybrid.

7. A nucleotide in accordance with item 1 wherein Sig is attached to B at a position such that when said nucleotide is incorporated into or attached to or associated with a double-stranded deoxyribonucleic acid or double-stranded ribonucleic acid or DNA-RNA hybrid, said chemical moiety Sig is capable of signalling itself or making itself self-detecting or its presence known.

8. An oligonucleotide or polydeoxyribonucleotide comprising one or more nucleotides in accordance with item 1.

9. An oligonucleotide or polyribonucleotide comprising one or more nucleotides in accordance with item 1.

10. A nucleotide in accordance with item 1 wherein said Sig is attached to said base B at the C5 position when said base B is a pyrimidine or at the C7 position when said base B is a deazapurin.

11. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is an aliphatic chemical moiety containing at least 4 carbon atoms.

12. A nucleotide in accordance with item 1 wherein said base B is a pyrimidine and wherein said Sig chemical moiety is attached to the pyrimidine at the N3 position.

13. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is an aliphatic chemical moiety containing at least 3 carbon atoms and at least one double bond.

14. A nucleotide in accordance with item 1 wherein said base B is a pyrimidine and wherein said Sig chemical moiety is attached to the pyrimidine at the C5 position.

15. A nucleotide in accordance with item 1 wherein said base B is a pyrimidine and wherein said Sig chemical moiety is attached to the pyrimidine at the C6 position.

16. A nucleotide in accordance with item 1 wherein said base B is a purine and wherein said Sig chemical moiety is attached to the purine at the N1 position.

17. A nucleotide in accordance with item 1 wherein said base B is a purine and wherein said Sig chemical moiety is attached to the purine at the C2 position.

18. A nucleotide in accordance with item 1 wherein said base B is a purine and wherein said Sig chemical moiety is attached to the purine at the N3 position.

19. A nucleotide in accordance with item 1 wherein said base B is a purine and wherein said Sig chemical moiety contains an aromatic or a cycloaliphatic group containing at least six or at least five carbon atoms, respectively.

20. A nucleotide in accordance with item 1 wherein said base B is a purine and wherein said Sig chemical moiety is attached to the purine at the N7 position.

21. A nucleotide in accordance with item 1 wherein said base B is a purine and wherein said Sig chemical moiety is attached to the purine at the C8 position.

22. A nucleotide in accordance with item 1 wherein said S sugar is a pentose.

23. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is a polysaccharide or an oligosaccharide.

24. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is a sugar selected from the group consisting of triose or tetrose, or pentose, a hexose, or heptose, and an octose.

25. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is attached by or includes a glycosidic linkage moiety.

26. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is a hexose moiety.

27. A nucleotide in accordance with item 1 wherein said base B is a 7-deazapurine and wherein said Sig chemical moiety is attached to the 7-deazapurine at the C7 position.

28. A nucleotide in accordance with item 1 wherein said Sig chemical moiety comprises a component selected from the group consisting of an electron dense component, a magnetic component, an enzyme, a hormone component, a radioactive component, a metal-containing component, a fluorescing component and an antigen or antibody component.

29. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is a sugar residue and wherein said sugar is complexed with or attached to a sugar or polysaccharide binding protein.

30. A nucleotide in accordance with item 29 wherein said protein is a lectin.

31. A nucleotide in accordance with item 30 wherein said lectin is Concanavalin A.

32. A nucleotide in accordance with item 1 wherein said Sig chemical moiety comprises a-mannosyl residue and wherein said a-mannosyl residue is complexed with or bound to Concanavalin A.

33. A nucleotide in accordance with item 1 wherein said Sig chemical moiety comprises N-acetylglucosamine residue and wherein N-acetylglucosamine is complexed with or bound to wheat germ agglutinin.

34. A nucleotide in accordance with item 1 wherein said Sig chemical moiety includes an electron dense component.

35. A nucleotide in accordance with item 30 wherein said lectin comprises ferritin attached thereto.

36. A nucleotide in accordance with item 31 wherein said Concanavalin A is conjugated to ferritin.

37. A nucleotide in accordance with item 1 wherein said Sig chemical moiety includes or comprises a radioactive isotope.

38. A nucleotide in accordance with item 37 wherein said radioactive isotope is radioactive cobalt.

39. A nucleotide in accordance with item 1 wherein said Sig chemical moiety includes or comprises an enzyme.

40. A nucleotide in accordance with item 39 wherein said enzyme is alkaline phosphatase.

41. A nucleotide in accordance with item 39 wherein said enzyme is acid phosphatase.

42. A nucleotide in accordance with item 1 wherein said Sig chemical moiety includes or comprises a fluorescing component attached thereto.

43. A nucleotide in accordance with item 42 wherein said fluorescing component is fluorescein.

44. A nucleotide in accordance with item 1 wherein said Sig chemical moiety includes or comprises a magnetic component associated or attached thereto.

45. A nucleotide in accordance with item 44 wherein said magnetic component comprises a magnetic oxide.

46. A nucleotide in accordance with item 45 wherein said magnetic oxide is ferric oxide.

47. A nucleotide in accordance with item 1 wherein said Sig chemical moiety includes an antigenic or hapten component capable of complexing with an antibody specific to said component.

48. A single-stranded polynucleotide comprising one or more nucleotides in accordance with item 1.

49. A single-stranded polynucleotide in accordance with item 48 wherein said single-stranded polynucleotide is a polydeoxyribonucleotide.

50. A single-stranded polynucleotide in accordance with item 48 wherein said single-stranded polynucleotide is a polyribonucleotide.

51. A double-stranded polynucleotide comprising one or more nucleotides in accordance with item 1.

52. A double-stranded polynucleotide in accordance with item 51 wherein said double-stranded polynucleotide is a double-stranded deoxyribonucleic acid.

53. A double-stranded polynucleotide in accordance with item 51 wherein said double-stranded polynucleotide is a double-stranded ribonucleic acid.

54. A double-stranded polynucleotide in accordance with item 51 wherein said double-stranded polynucleotide is a double-stranded deoxyribonucleic acid-ribonucleic acid hybrid.

55. A polynucleotide comprising one or more nucleotides in accordance with item 1 coupled or attached to a polypeptide. said polypeptide having attached thereto one or more biotin groups.

56. A polynucleotide in accordance with item 55 wherein said polypeptide is a polylysine.

57. A polynucleotide coupled or attached to a polypeptide. said polypeptide having attached thereto one or more streptavidin units.

58. A polynucleotide terminally ligated or attached on at least one end with a polypeptide. said polypeptide having attached thereto one or more enzyme groups.

59. A single stranded polydeoxyribonucleotide coupled or attached to a polypeptide. said polypeptide having attached thereto one or more biotin groups.

60. A single-stranded polyribonucleotide coupled or attached to a polypeptide. said polypeptide having attached thereto one or more biotin groups.

61. A single-stranded polynucleotide comprising at least 12 nucleotides, at least one of said nucleotides being a nucleotide in accordance with item 1.

62. A polynucleotide coupled or attached to a polysaccharide.

63. A polynucleotide in accordance with item 62 wherein said polysaccharide has attached thereto or complexed therewith a plant binding protein.

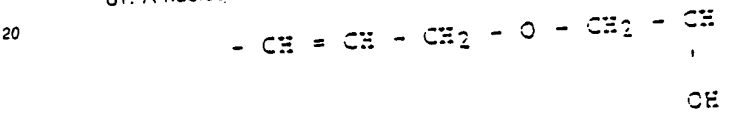
64. A polynucleotide in accordance with item 63 wherein said protein is Concanavalin A.

65. A nucleotide in accordance with item 1 wherein said base B is cytosine.

66. A nucleotide in accordance with item 1 wherein said base B is uracil.

67. A nucleotide in accordance with item 1 wherein said base B is thymine.

68. A nucleotide in accordance with item 1 wherein said base B is adenine.
69. A nucleotide in accordance with item 1 wherein said base B is guanine.
70. A nucleotide in accordance with item 1 wherein said base B is 2-methyladenine.
71. A nucleotide in accordance with item 1 wherein said base B is 1-methylguanine.
72. A nucleotide in accordance with item 1 wherein said base B is 5-methylcytosine.
73. A nucleotide in accordance with item 1 wherein said base B is 5-hydroxymethylcytosine.
74. A nucleotide in accordance with item 1 wherein said Sig chemical moiety comprises a chelating agent.
75. A nucleotide in accordance with item 1 wherein said base B is deazaadenine.
76. A nucleotide in accordance with item 1 wherein said base B is deazaguanine.
77. A nucleotide in accordance with item 1 wherein said Sig chemical moiety is connected to said base B via a chemical linkage.
78. A nucleotide in accordance with item 77 wherein said chemical linkage includes an olefinic bond at the a-position relative to base B.
79. A nucleotide in accordance with item 77 wherein said chemical linkage includes the moiety, $-CH_2-NH-$.
80. A nucleotide in accordance with item 77 wherein said chemical linkage is, $-CH=CH-CH_2-NH-$.
81. A nucleotide in accordance with item 1 wherein the chemical moiety is,



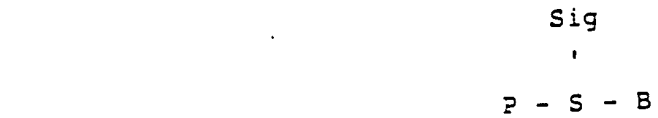
82. A nucleotide in accordance with item 1 wherein said chemical linkage is selected from or includes a moiety selected from the group consisting of

83. A nucleotide in accordance with item 1 wherein said Sig chemical moiety includes or comprises a catalytic metal component.
84. A nucleotide in accordance with item 39 wherein said enzyme is β-galactosidase.
85. A nucleotide in accordance with item 39 wherein said enzyme is glucose oxidase.
86. A nucleotide in accordance with item 39 wherein said enzyme is horseradish peroxidase.
87. A nucleotide in accordance with item 42 wherein said fluorescing component is fluorescein.
88. A nucleotide in accordance with item 42 wherein said fluorescing component is rhodamine.
89. A nucleotide in accordance with item 42 wherein said fluorescing component is dansyl.
90. A nucleotide in accordance with item 80 wherein said Sig chemical moiety is attached to the - NH - group of said chemical linkage.
91. A nucleotide in accordance with item 90 wherein said Sig chemical moiety comprises a polysaccharide.
92. A nucleotide in accordance with item 90 wherein said Sig chemical moiety is biotin.
93. A nucleotide in accordance with item 90 wherein said Sig chemical moiety is streptavidin.
94. A nucleotide in accordance with item 82 wherein said Sig chemical moiety attached to said chemical linkage is a monosaccharide.
95. A nucleotide in accordance with item 82 wherein said Sig chemical moiety attached to said chemical linkage is a streptavidin.
96. A polynucleotide in accordance with item 63 wherein said protein is a lectin.
97. A polynucleotide in accordance with item 62 wherein said polynucleotide is terminally ligated to said polysaccharide.
98. A polyribonucleotide in accordance with item 60 wherein said polyribonucleotide is terminally ligated or attached to said polypeptide.

99. A polydeoxyribonucleotide in accordance with item 59 wherein said polydeoxyribonucleotide is terminally ligated or attached to said polypeptide.

100. A polynucleotide in accordance with item 55 wherein said polynucleotide is terminally ligated or attached to said polypeptide.

101. A ribonucleotide having the general formula,



wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety, the phosphoric acid moiety being attached at the 2', 3' and or 5' position of the sugar moiety, said base B being attached from the N1 position or the N9 position to the 1' position of the sugar moiety when said base is a pyrimidine or a purine, respectively, and wherein said Sig is a chemical moiety covalently attached to the sugar S, said Sig, when attached to said sugar S, being capable of signalling itself or making itself self-detecting or its presence known.

102. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety is attached to the C2' position of said sugar moiety.

103. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety is attached to the C3' position of said sugar moiety.

104. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety is attached to the S sugar moiety such that an oligoribonucleotide or polyribonucleotide containing said ribonucleotide is capable of forming a double-stranded ribonucleic acid or a DNA-RNA hybrid when said ribonucleotide is incorporated into said oligoribonucleotide or said polyribonucleotide.

105. A polyribonucleotide comprising at least one ribonucleotide in accordance with item 101.

106. A ribonucleotide in accordance with item 1 wherein said base B is a pyrimidine.

107. A ribonucleotide in accordance with item 101 wherein said base B is a purine.

108. A ribonucleotide in accordance with item 101 wherein said base B is uracil.

109. A ribonucleotide in accordance with item 101 wherein said base B is adenine.

110. A ribonucleotide in accordance with item 101 wherein said base B is guanine.

111. A ribonucleotide in accordance with item 101 wherein said base B is cytosine.

112. A ribonucleotide in accordance with item 101 wherein said base B is a 7-deazapurine.

113. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety is a polysaccharide or an oligosaccharide.

114. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety is a monosaccharide.

115. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety is a monosaccharide selected from the group consisting of triose, tetrose, pentose, hexose, heptose and octose.

116. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety is a sugar residue and wherein said sugar residue is complexed with or attached to a sugar or polysaccharide binding protein.

117. A ribonucleotide in accordance with item 116 wherein said protein is a lectin.

118. A ribonucleotide in accordance with item 117 wherein said lectin is Concanavalin A.

119. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety includes an electron dense component.

120. A ribonucleotide in accordance with item 119 wherein said electron dense component is ferritin.

121. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety includes or comprises a radioactive isotope component.

122. A ribonucleotide in accordance with item 121 wherein said radioactive isotope component is radioactive cobalt.

123. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety comprises a catalytic metal component.

124. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety includes or comprises an enzyme.

125. A ribonucleotide in accordance with item 124 wherein said enzyme is alkaline phosphatase.

126. A ribonucleotide in accordance with item 124 wherein said enzyme is β-galactosidase.

127. A ribonucleotide in accordance with item 124 wherein said enzyme is glucose oxidase.

128. A ribonucleotide in accordance with item 124 wherein said enzyme is horseradish peroxidase.

129. A ribonucleotide in accordance with item 124 wherein said enzyme is ribonuclease.

130. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety includes or comprises a floresching component attached thereto.

131. A ribonucleotide in accordance with item 130 wherein said fluorescing component is fluoroscein.

132. A ribonucleotide in accordance with item 130 wherein said fluorescing component is rhodamine.

133. A ribonucleotide in accordance with item 130 wherein said fluorescing component is dansyl.

134. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety comprises a magnetic component associated or attached thereto.

135. A ribonucleotide in accordance with item 134 wherein said magnetic component comprises a magnetic oxide.

136. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety includes an antigenic or hapten component capable of complexing with an antibody specific to said component.

137. A single stranded polyribonucleotide comprising one or more ribonucleotides in accordance with item 101, said single stranded polyribonucleotide comprising at least three said ribonculeotides.

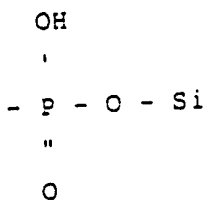
138. A single stranded polyribonucleotide comprising at least twelve ribonucleotides and containing at least one ribonucleotide in accordance with item 101.

139. A polyribonucleotide coupled or attached to a polypeptide.

140. A polyribonucleotide in accordance with item 139 wherein said polypeptide is terminally attached or ligated to said polyribonucleotide.

141. A nucleotide having the general formula

wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety, the phosphoric acid moiety being attached to the 3' and/or the 5' position of the sugar moiety when said nucleotide is a deoxyribonucleotide and at the 2', 3' and/or 5' position when said nucleotide is a ribonucleotide, said base B being a purine or pyrimidine, said base B being attached from the N1 position or the N9 position to the 1' position of the sugar moiety when said base B is a pyrimidine or a purine, respectively, and wherein Sig is a chemical moiety covalently attached to the phosphoric acid moiety via the chemical linkage



said Sig, when attached to said phosphoric acid moiety P being capable of signalling itself or making itself self-detecting or its presence known.

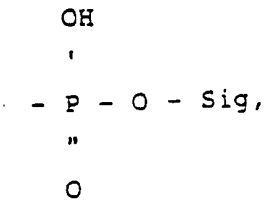
142. A nucleotide having the general formula

P - S - B - Sig

wherein P is the phosphoric acid moiety, S the sugar and monosaccharide moiety, B being the base moiety, the phosphoric acid moiety being attached to the 3' and/or the 5' position of the sugar moiety when said nucleotide is deoxyribonucleotide and at the 2', 3' and/or 5' position when said nucleotide is a ribonucleotide, said base being a purine or a pyrimidine, said base being attached from the N1 position or the N9 position to the C1' position of the sugar moiety when said base is a pyrimidine or a purine, respectively, and wherein said Sig is a chemical moiety covalently attached to the base B of said nucleotide, said Sig being attached to the N^6 or 6-amino group when said base B is adenine or the N^2 or 2-

amino group when said base B is guanine or the N^4 or 4-amino group when said base B is cytosine, said Sig when attached to said base B being capable of signally itself or makes itself self-detecting or its presence known.

143. A nucleotide having the general formula P-S-B, wherein P is the phosphoric acid moiety, S the sugar or monosaccharide moiety and B the base moiety, said nucleotide having covalently attached to the P or S or B moiety a chemical moiety Sig, said Sig chemical moiety when attached to the P moiety is attached thereto via the chemical linkage,



and when Sig is attached to the S moiety, the S moiety is a ribose group, said chemical moiety Sig when attached to said P, S or B being capable of signalling itself or makes itself self-detecting or its presence known.

144. A nucleotide in accordance with item 143 wherein said nucleotide is capable of being incorporated into to form a double-stranded ribonucleic acid, a double-stranded deoxyribonucleic acid or a double-stranded deoxyribonucleic acid-ribonucleic acid hybrid.

145. A nucleotide in accordance with item 143 wherein when said nucleotide is incorporated into or attached to a double-stranded deoxyribonucleic acid or double-stranded ribonucleic acid or DNA-RNA hybrid, said chemical moiety Sig is capable of signalling itself or making itself self-detecting or its presence known.

146. A single-stranded polynucleotide comprising one or more nucleotides in accordance with item 143.

147. A double-stranded polynucleotide comprising one or more nucleotides in accordance with 143.

148. A single-stranded polydeoxyribonucleotide containing at least 12 nucleotides and comprising one or more nucleotides in accordance with item 143.

149. A nucleotide in accordance with Claim 143 wherein said Sig moiety is attached to the B base moiety through a mono-or oligosaccharide linkage.

150. A nucleotide in accordance with item 143 wherein said Sig moiety is monosaccharide or polysaccharide moiety attached to said B base moiety.

151. A polynucleotide comprising at least one nucleotide in accordance with item 143.

152. A polynucleotide comprising at least one nucleotide in accordance with item 149.

153. A polynucleotide comprising at least one nucleotide in accordance with item 150.

154. A nucleotide in accordance with Claim 143 or polynucleotide comprising at least one nucleotide in accordance with item 143 wherein said Sig chemical moiety comprises an agent for stimulating or inducing the production of interferon.

155. A method of chemotherapy suitable for inhibiting RNA and/or DNA synthesis which comprises administering to an organism capable of and/or functioning for the production or synthesis of DNA and/or RNA an effective DNA and/or RNA synthesis inhibiting amount of a nucleotide in accordance with item 143.

156. A method of chemotherapy suitable for inhibiting RNA and/or DNA synthesis which comprises administering to an organism capable of and/or functioning for the production or synthesis of DNA and/or RNA an effective DNA and/or RNA synthesis inhibiting amount of a nucleotide in accordance with item 1.

157. A method of chemotherapy suitable for inhibiting RNA and/or DNA synthesis which comprises administering to the organism capable of and/or functioning for the production or synthesis of DNA and/or RNA an effective DNA and/or RNA synthesis inhibiting amount of a nucleotide in accordance with item 101.

158. A method of chemotherapy suitable for inhibiting RNA and/or DNA synthesis which comprises administering to the organism capable of and/or functioning for the production or synthesis of DNA and/or RNA an effective DNA and/or RNA synthesis inhibiting amount of a nucleotide in accordance with item 141.

159. A method of chemotherapy suitable for inhibiting RNA and/or DNA synthesis which comprises administering to the organism capable of and/or functioning for the production or synthesis of DNA and/or RNA an effective DNA and/or RNA synthesis inhibiting amount of a nucleotide in accordance with item 142.

160. A method of chemotherapy in accordance with item 155 wherein the B base moiety of said nucleotide is glycosylated.

161. A method of chemotherapy in accordance with item 155 wherein the Sig chemical moiety of said nucleotide comprises an anti-tumor or cytotoxic agent.

162. A method for the stimulation or induction of cells for the production of lymphokines, cytokinins and/or interferon which comprises introducing into or bringing into contact with cells capable of and/or functioning for the production of said lymphokines, cytokinins and/or interferon an effective lymphokine, cytokinin and/or interferon stimulating and production inducing amount of a nucleotide in accordance with item 143.

163. A method for the stimulation or induction of cells for the production of lymphokines, cytokinins and/or interferon which comprises introducing into or bringing into contact with cells capable of and/or functioning for the production of said lymphokines, cytokinins and/or interferon an effective lymphokine, cytokinin and/or interferon stimulating and production inducing amount of a nucleotide in accordance with item 1.

164. A method for the stimulation or induction of cells for the production of lymphokines, cytokinins and/or interferon which comprises introducing into or bringing into contact with cells capable of and/or functioning for the production of said lymphokines, cytokinins and/or interferon an effective lymphokine, cytokinin and/or interferon stimulating and production inducing amount of a nucleotide in accordance with item 101.

165. A method for the stimulation or induction of cells for the production of lymphokines, cytokinins and/or interferon which comprises introducing into or bringing into contact with cells capable of and/or functioning for the production of said lymphokines, cytokinins and/or interferon an effective lymphokine, cytokinin and/or interferon stimulating and production inducing amount of a nucleotide in accordance with item 141.

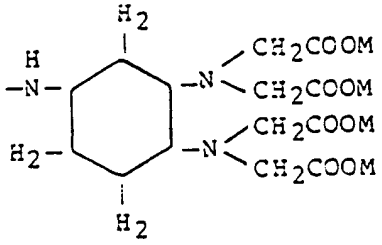
166. A method for the stimulation or induction of cells for the production of lymphokines, cytokinins and/or interferon which comprises introducing into or bringing into contact with cells capable of and/or functioning for the production of said lymphokines, cytokinins and/or interferon an effective lymphokine, cytokinin and/or interferon stimulating and production inducing amount of a nucleotide in accordance with item 142.

167. A polynucleotide comprising one or more nucleotides in accordance with item 1 or item 101 or item 141 or item 142 or item 143, coupled or attached to a polypeptide, said polypeptide having attached thereto one or more Sig chemical moieties, said Sig when attached to said polypeptide being capable of signalling itself or making itself self-detecting or its presence known.

168. A polynucleotide coupled or attached to a polypeptide, said polypeptide having attached thereto one or more Sig chemical moieties, said Sig chemical moieties when attached to said polypeptide being capable of signalling itself or making itself self-detecting or making its presence known.

169. A nucleotide in accordance with item 1 or item 101 or item 141 or item 142 or item 143 wherein said Sig chemical moiety includes a chelating agent.

170. A nucleotide in accordance with item 169 wherein said chelating agent includes the chemical moiety



wherein M is H or a substitutable metal.

171. A nucleotide in accordance with item 170 wherein said metal is magnesium or a metal replaceable by cobalt.

172. A polynucleotide containing one or more nucleotides in accordance with item 1 or item 101 or item 141 or item 142 or item 143 wherein said Sig chemical moiety includes a chelating agent.

173. A polynucleotide containing a nucleotide in accordance with item 1 or item 101 or item 141 or item 142 or item 143 wherein said Sig chemical moiety includes a chelating agent in accordance with Claim 170.

174. A nucleotide in accordance with item 170 wherein said metal is a catalytically active metal.

175. A nucleotide in accordance with item 170 wherein said metal is a heavy metal.

176. A nucleotide in accordance with item 170 wherein said metal is radioactive cobalt.

177. A nucleotide in accordance with item 170 wherein said metal is magnesium.

178. A ribonucleotide in accordance with item 1 or item 101 or item 141 or item 142 or item 143 wherein said Sig chemical moiety includes a chelating agent.

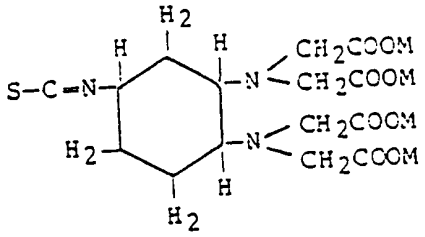
179. A deoxyribonucleotide in accordance with item 1 or item 101 item 141 or item 142 or item 143 wherein said Sig chemical moiety includes a chelating agent.

180. A nucleotide in accordance with item 170 wherein said metal M is a radioactive isotope.

181. A nucleotide in accordance with item 170 wherein said metal is platinum.

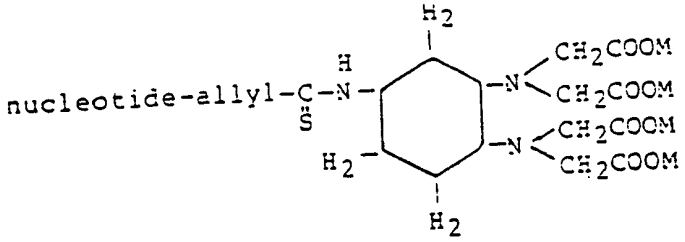
182. A nucleotide in accordance with item 170 wherein said M is hydrogen or a substitutable metal or a radioactive element.

183. The compound



wherein M is hydrogen or a metal.

184. The compound

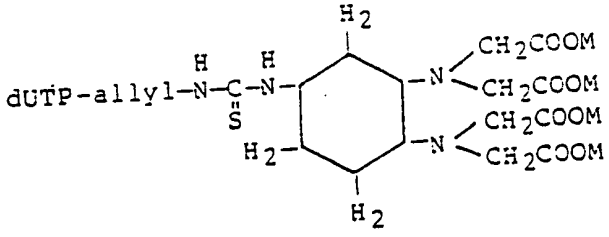


wherein M is hydrogen or a metal.

185. A compound in accordance with item 184 wherein said nucleotide is a deoxyribonucleotide.

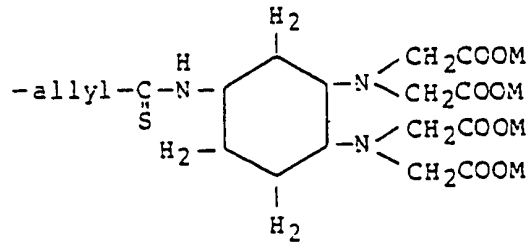
186. A compound in accordance with item 184 wherein said nucleotide is a ribonucleotide.

187. The compound



wherein M is hydrogen or a metal.

188. A nucleotide in accordance with items 1, 101, 141, 142 or 143, wherein the Sig chemical moiety comprises the chemical moiety



189. A polynucleotide containing one or more nucleotides in accordance with item 188.

190. A polynucleotide in accordance with item 189 wherein said polynucleotide is a single-stranded deoxyribonucleotide or a single-stranded ribonucleotide or a double-stranded DNA, RNA or DNA-RNA hybrid.

191. A nucleotide in accordance with item 143 wherein said Sig moiety comprises a saccharide component, a protein component capable of binding to said saccharide component and a glycosylated enzyme component capable of binding to said protein component.

192. A nucleotide in accordance with item 191 wherein said saccharide component is a monosaccharide.

193. A nucleotide in accordance with item 191 wherein said saccharide component is an oligosaccharide.

194. A nucleotide in accordance with item 191 wherein said saccharide component is a polysaccharide.

195. A nucleotide in accordance with item 191 wherein said protein component is a lectin.

196. A nucleotide in accordance with item 191 wherein said lectin is a plant lectin selected from the group consisting of Concanavalin A, LCH lentil lectin, PSA pea lectin and BFA Vicia Faba lectin.

197. A nucleotide in accordance with item 191 wherein said enzyme component is an enzyme selected from the group consisting of alkaline phosphatase, acid phosphatase and horseradish peroxidase.

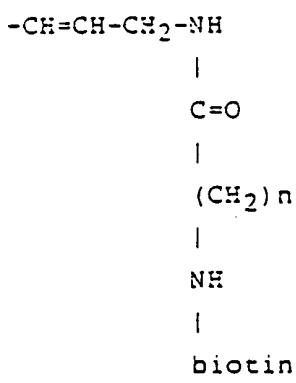
198. An amino acid or polypeptide comprising a Sig moiety attached thereto.

199. An amino acid or polypeptide in accordance with item 198 wherein said Sig moiety comprises a saccharide component.

200. A monosaccharide or a polysaccharide comprising a Sig moiety attached thereto.

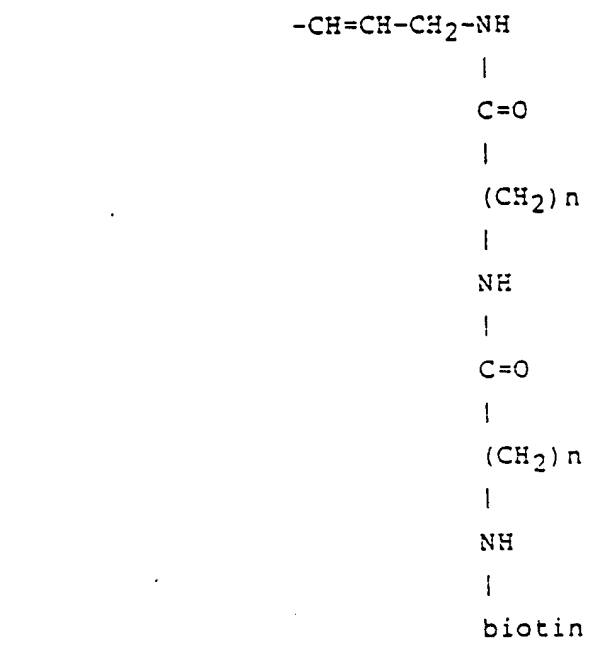
201. A monosaccharide or a polysaccharide in accordance with item 200 wherein said Sig moiety comprises a chelating agent.

202. A nucleotide in accordance with item 143 wherein said Sig moiety comprises the linkage



wherein n is an integer having a value in the range 2-10.

203. A nucleotide in accordance with item 143 wherein said Sig moiety comprises the linkage



wherein n is an integer having a value in the range 2-10.

204. A method of detecting a first compound which includes a nucleotide in accordance with item 1 as part of said first compound, which comprises contacting said first compound with a second compound capable of forming a complex therewith under suitable conditions so as to form said complex, said complex comprising said first compound and said second compound and detecting said complex.

205. A method in accordance with item 204 wherein said second compound is a polynucleotide.

206. A method of detecting a first compound which includes a ribonucleotide in accordance with item 101 as part of said first compound, which comprises contacting said first compound with a second compound capable of forming a complex therewith under conditions so as to form said complex and detecting said complex.

207. A method of detecting a first compound which includes a nucleotide in accordance with item 141 as part of said first compound which comprises contacting said first compound with a second compound capable of forming a complex therewith under suitable conditions so as to form said complex and detecting said complex.

208. A method of detecting a first compound which includes a nucleotide in accordance with item 142 as part of said first compound which comprises contacting said first compound with a second compound capable of forming a complex therewith under suitable conditions so as to form said complex and contacting said complex.

209. A method of detecting a first compound which includes a nucleotide in accordance with item 143 as part of said first compound which comprises contacting said first compound with a second compound capable of forming a complex therewith under suitable conditions so as to form said complex and detecting said complex.

210. A method of determining the presence of a deoxyribonucleic or ribonucleic acid molecule which comprises forming a double-stranded hybrid polynucleotide duplex which includes a single strand of deoxyribonucleic acid or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid molecule and a nucleotide in accordance with item 1 and detecting said duplex.

211. A method of determining the presence of a deoxyribonucleic or ribonucleic acid molecule which comprises forming a double-stranded hybrid polynucleotide duplex which includes a single strand of deoxyribonucleic acid or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid molecule and a ribonucleotide in accordance with item 101 and detecting said duplex.

212. A method of determining the presence of a deoxyribonucleic or ribonucleic acid molecule which comprises forming a double-stranded hybrid polynucleotide duplex which includes a single strand of deoxyribonucleic acid or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid molecule and a nucleotide in accordance with item 141 and detecting said duplex.

213. A method of determining the presence of a deoxyribonucleic or ribonucleic acid molecule which comprises forming a double-stranded hybrid polynucleotide duplex which includes a single strand of deoxyribonucleic acid or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid molecule and a nucleotide in accordance with item 142 and detecting said duplex.

214. A method of determining the presence of a deoxyribonucleic or ribonucleic acid molecule which comprises forming a double-stranded hybrid polynucleotide duplex which includes a single strand of deoxyribonucleic acid or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid molecule and a nucleotide in accordance with item 143 and detecting said duplex.

215. A method of detecting the presence of a nucleic acid-containing etiological agent in a subject which comprises obtaining a suitable sample from said subject, detecting the presence in said sample of a deoxyribonucleic or ribonucleic acid naturally associated with said etiological agent by forming under suitable conditions a double-stranded polynucleotide duplex which includes a compound in accordance with item 1 and a single strand of deoxyribonucleic or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid which is naturally associated with said etiological agent under suitable conditions and detecting the presence of said double-stranded polynucleotide duplex.

216. A method of detecting the presence of a nucleic acid-containing etiological agent in a subject which comprises obtaining a suitable sample from said subject, detecting the presence in said sample of a deoxyribonucleic or ribonucleic acid naturally associated with said etiological agent by forming under suitable conditions a double-stranded polynucleotide duplex which includes a compound in accordance with item 101 and a single strand of dexoyribonucleic or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid which is naturally associated with said etiological agent under suitable conditions and detecting the presence of said double-stranded polynucleotide duplex.

217. A method of detecting the presence of a nucleic acid-containing etiological agent in a subject which comprises obtaining a suitable sample from said subject, detecting the presence in said sample of a deoxyribonucleic or ribonucleic acid naturally associated with said etiological agent by forming under suitable conditions a double-stranded polynucleotide duplex which includes a compound in accordance with item 141 and a single strand of deoxyribonucleic or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid which is naturally associated with said etiological agent under suitable conditions and detecting the presence of said double-stranded polynucleotide duplex.

218. A method of detecting the presence of a nucleic acid-containing etiological agent in a subject which comprises obtaining a suitable sample from said subject, detecting the presence in said sample of a deoxyribonucleic or ribonucleic acid naturally associated with said etiological agent by forming under suitable conditions a double-stranded polynucleotide duplex which includes a compound in accordance with item 142 and a single strand of deoxyribonucleic or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid which is naturally associated with said etiological agent under suitable conditions and detecting the presence of said double-stranded polynucleotide duplex.

219. A method of detecting the presence of a nucleic acid-containing etiological agent in a subject which comprises obtaining a suitable sample from said subject, detecting the presence in said sample of a deoxyribonucleic or ribonucleic acid naturally associated with said etiological agent by forming under suitable conditions a double-stranded polynucleotide duplex which includes a compound in accordance with item 143 and a single strand of deoxyribonucleic or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid which is naturally associated with said etiological agent under suitable conditions and detecting the presence of said double-stranded polynucleotide duplex.

220. A method in accordance with item 215 wherein said nucleic acid-containing etiological agent is a bacterium, a virus or a fungus.

221. A method of testing a bacterium to determine its resistance to an antibiotic which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said bacterium which confers resistance of said bacterium to said antibiotic and which includes a nucleotide in accordance with item 1 incorporated therein, contacting said polynucleotide under suitable conditions with a deoxyribonucleic acid obtained from said bacterium so as to form a double-stranded hybrid duplex and detecting the presence of said duplex, the detection of said duplex indicating resistance of said bacterium to said antibiotic and the absence of said duplex indicating the susceptibility of said bacterium to an antibiotic.

222. A method of testing a bacterium to determine its resistance to an antibiotic which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said bacterium which confers resistance of said bacterium to said antibiotic and which includes a nucleotide in accordance with item 101 incorporated therein, contacting said polynucleotide under suitable conditions with a deoxyribonucleic acid obtained from said bacterium so as to form a double-stranded hybrid duplex and

detecting the presence of said duplex, the detection of said duplex indicating resistance of said bacterium to said antibiotic and the absence of said duplex indicating the susceptibility of said bacterium to an antibiotic.

223. A method of testing a bacterium to determine its resistance to an antibiotic which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said bacterium which confers resistance of said bacterium to said antibiotic and which includes a nucleotide in accordance with item 141 incorporated therein, contacting said polynucleotide under suitable conditions with a deoxyribonucleic acid obtained from said bacterium so as to form a double-stranded hybrid duplex and detecting the presence of said duplex, the detection of said duplex indicating resistance of said bacterium to said antibiotic and the absence of said duplex indicating the susceptibility of said bacterium to an antibiotic.

224. A method of testing a bacterium to determine its resistance to an antibiotic which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said bacterium which confers resistance of said bacterium to said antibiotic and which includes a nucleotide in accordance with (item 142 incorporated therein, contacting said polynucleotide under suitable conditions with a deoxyribonucleic acid obtained from said bacterium so as to form a double-stranded hybrid duplex and detecting the presence of said duplex, the detection of said duplex indicating resistance of said bacterium to said antibiotic and the absence of said duplex indicating the susceptibility of said bacterium to an antibiotic.

225. A method of testing a bacterium to determine its resistance to an antibiotic which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said bacterium which confers resistance of said bacterium to said antibiotic and which includes a nucleotide in accordance with item 143 incorporated therein, contacting said polynucleotide under suitable conditions with a deoxyribonucleic acid obtained from said bacterium so as to form a double-stranded hybrid duplex and detecting the presence of said duplex, the detection of said duplex indicating resistance of said bacterium to said antibiotic and the absence of said duplex indicating the susceptibility of said bacterium to an antibiotic.

226. A method in accordance with items 221, 222, 223, 224 or 225 wherein said bacterium is selected from the group consisting of Streptococcus pyogenes and Neisseria meningitidis and said antbiotic a penicillin.

227. A method in accordance with item 221 wherein said bacterium is selected from the group consisting of Staphylococcus aureus, Candida albicans, Pseudomonas aeruginosa, Streptococcus pyogenes and Neisseria gonorrhoeae and said antibiotic a tetracycline.

228. A method in accordance with item 221 wherein said bacterium is Mycobacterium tuberculosis and said antibiotic is an amino glycoside.

229. A method of diagnosing a genetic disorder in a subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said subject which is associated with said genetic disorder and includes a compound in accordance with item 1 incorporated therein, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and detecting the presence of said hybrid duplex, the presence or absence of said hybrid duplex indicating the presence or absence of said genetic disorder.

230. A method of diagnosing a genetic disorder in a subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said subject which is associated with said genetic disorder and includes a compound in accordance with item 101 incorporated therein, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and detecting the presence of said hybrid duplex, the presence or absence of said hybrid duplex indicating the presence or absence of said genetic disorder.

231. A method of diagnosing a genetic disorder in a subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said subject which is associated with said genetic disorder and includes a compound in accordance with item 141 incorporated therein, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and detecting the presence of said hybrid duplex, the presence or absence of said hybrid duplex indicating the presence or absence of said genetic disorder.

232. A method of diagnosing a genetic disorder in a subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said subject which is associated with said genetic disorder and includes a compound in accordance with item 142 incorporated therein,

contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and detecting the presence of said hybrid duplex, the presence or absence of said hybrid duplex indicating the presence or absence of said genetic disorder.

233. A method of diagnosing a genetic disorder in a subject which comprises preparing a polynucleotide complementary to the deoxyribonucleotide acid gene sequence of said subject which is associated with said genetic disorder and includes a compound in accordance with item 143 incorporated therein, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and detecting the presence of said hybrid duplex, the presence or absence of said hybrid duplex indicating the presence or absence of said genetic disorder.

234. A method of diagnosing thalassemia in a human subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence which is absent in β-minus-thalassemia subjects and includes a nucleotide in accordance with item 1, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and determining the presence of said duplex, the absence of said duplex indicating the presence of β-minus-thalassemia.

235. A method of diagnosing thalassemia in a human subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence which is absent in β-minus-thalassemia subjects and includes a nucleotide in accordance with item 101, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and determining the presence of said duplex, the absence of said duplex indicating the presence of β-minus-thalassemia.

236. A method of diagnosing thalassemia in a human subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence which is absent in β-minus-thalassemia subjects and includes a nucleotide in accordance with item 141, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and determining the presence of said duplex, the absence of said duplex indicating the presence of β-minus-thalassemia.

237. A method of diagnosing thalassemia in a human subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence which is absent in β-minus-thalassemia subjects and includes a nucleotide in accordance with item 142, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and determining the presence of said duplex, the absence of said duplex indicating the presence of β-minus-thalassemia.

238. A method of diagnosing thalassemia in a human subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence which is absent in β-minus-thalassemia subjects and includes a nucleotide in accordance with item 143, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and determining the presence of said duplex, the absence of said duplex indicating the presence of β-minus-thalassemia.

239. A method of chromosomal karyotyping which comprises preparing a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, said polynucleotides including one or more compounds in accordance with item 1, contacting said polynucleotides with deoxyribo nucleic acid of or obtained from chromosomes so as to form hybrid duplexes and detecting said duplexes, thereby determining the location of said duplexes on said chromosomes and the location of said genetic sequences on said chromosomes.

240. A method of chromosomal karyotyping which comprises preparing a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, said polynucleotides including one or more compounds in accordance with item 101, contacting said polynucleotides with deoxyribonucleic acid of or obtained from chromosomes so as to form hybrid duplexes and detecting said duplexes, thereby determining the location of said duplexes on said chromosomes and the location of said genetic sequences on said chromosomes.

241. A method of chromosomal karyotyping which comprises preparing a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, said polynucleotides including one or more compounds in accordance with item 141 contacting said polynucleotides with deoxyribonucleic acid of or obtained from chromosomes so as to form hybrid duplexes and detecting said duplexes, thereby determining the location of said duplexes on said chromosomes and the location of said genetic sequences on said chromosomes.

242. A method of chromosomal karyotyping which comprises preparing a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, said polynucleotides including one or more compounds in accordance with item 142, contacting said polynucleotides with deoxyribonucleic acid of or obtained from chromosomes so as to form hybrid duplexes and detecting said duplexes, thereby determining the location of said duplexes on said chromosomes and the location of said genetic sequences on said chromosomes.

243. A method of chromosomal karyotyping which comprises preparing a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, said polynucleotides including one or more compounds in accordance with item 143, contacting said polynucleotides with deoxyribonucleic acid of or obtained from chromosomes so as to form hybrid duplexes and detecting said duplexes, thereby determining the location of said duplexes on said chromosomes and the location of said genetic sequences on said chromosomes.

244. A method of identifying or locating hormone receptor sites on the surface of cells which comprises binding a compound in accordance with items 1, 101, 141, 142 or 143 to said sites under suitable conditions permitting binding of said compound to said receptor sites and detecting said compounds bound to said receptor sites.

245. A method of tumor or cancer cell identification or detection which comprises identifying or detecting malignant cells by detecting abnormal receptor sites associated therewith in accordance with item 244.

246. A method of diagnosing a tumor cell which comprises preparing a polynucleotide which is complementary to a messenger ribonucleic acid synthesized from a deoxyribonucleic acid gene sequence associated with the production of a polypeptide diagnostic for or identifiable with said tumor cell or which is complementary to said deoxyribonucleic acid gene sequence and which includes a compound in accordance with items 1, 101, 141, 142 or 143, introducing said polynucleotide into contact with said cell under suitable conditions so as to permit said polynucleotide to hybridize with said deoxyribonucleic acid gene sequence or said messenger ribonucleic acid and detecting the formation of a resulting formed hybrid containing said polynucleotide.

247. A diagnostic kit useful for determining the presence of a nucleic acid-containing organism or the like which comprises a polynucleotide which includes in its make-up a compound selected from the compounds of items 1, 101, 141, 142 or 143, and which is complementary to all or an identifiable, distinct or unique portion of the nucleic acid contained in said organism and means for detecting or expressing the presence or absence of a resulting formed hybrid between said polynucleotide and said nucleic acid of said organism when said polynucleotide is brought into contact with the nucleic acid of said organism or the like under hybrid forming conditions.

248. A method of determining or identifying or diagnosing a first compound capable of complexing with or binding with a first component of a second compound, said second compound comprising said first component and a second component, said second component being attached to or complexed with said first component, which comprises bringing said first compound into contact with said second compound whereby said first component of said second compound complexes with said first compound to form a third compound comprising said first compound and said second compound and contacting said resulting third compound with a fourth compound capable of binding with or attaching to said second component of said second compound making up said third compound.

249. A method in accordance with item 248 wherein multiple amounts of said second compound are separately brought into contact with said first compound or vice versa.

250. A method in accordance with item 248 wherein said first compound is a molecule with a receptor site that specifically binds to a second molecule, wherein said first component of said second compound is capable of specifically binding with said receptor site of said first compound, wherein said second component of said second compound is a sugar, oligosaccharide or polysaccharide and wherein said fourth compound is a polypeptide.

251. A method of determining or identifying or diagnosing a first compound capable of complexing with or binding with an amino acid, peptide or protein which comprises bringing said first compound into contact with a second compound, said second compound comprising an amino acid, peptide or protein attached to or complexed with a sugar, or oligosaccharide or polysaccharide, whereupon said second compound complexes with or becomes attached to said first compound to form a third compound comprising said first compound and said second compound and contacting said resulting third compound with a protein or amino acid or peptide capable of binding with or attaching to the sugar or oligosaccharide moiety of said second compound making up said third compound.

252. A method of determining or identifying or diagnosing a first compound capable of complexing or binding with a first component of a second compound, said second compound comprising said first component, a second component attached to or complexed with said first component, said second compound being a chelating agent, and a third component, said third component being an ion chelated or fixed to said second component, which comprises bringing said first compound into contact with said second compound whereby said first component of said compound complexes with said first compound to form a third compound comprising said first compound and said second compound.

253. A method in accordance with item 252 wherein said third compound is brought into contact with or reacted with a fourth compound under conditions such that said third component of said second compound making up said third compound catalyzes a discernible or detectable reaction involving or otherwise interacting with said fourth compound.

254. A method of chemotherapy involving the delivery of a therapeutic or cytotoxic agent to a selected receptor site of an organism or human subject which comprises bringing a first compound comprising a first component, a second component and a third component into contact with said receptor site, said first component being capable of attaching to or complexing with said receptor site, said second component being attached to said first component and said third component comprising a therapeutic or cytotoxic agent attached to said second component whereby upon contacting said receptor site with said first compound, said therapeutic or cytotoxic agent brought into contact with or delivered to said receptor site, said second component being a sugar, oligosaccharide or polypeptide and said third component containing said therapeutic or cytotoxic agent additionally comprising an amino acid or peptide or protein attached to or fixed to said second component.

255. A method of determining or identifying or diagnosing in an organism, cellular material or tissue a first compound comprising a receptor molecule with a receptor site that specifically binds to a second molecule which comprises bringing said organism, material or tissue containing said receptor molecule into contact with a second compound, said second compound comprising a first component capable of complexing with or binding with said receptor molecule, said second compound also comprising a second component, said second component being attached to or complexed with said first component, said first component of said second compound when in contact with said receptor molecule complexes with said receptor molecule to form a third compound comprising said first compound and said second compound and contacting said third compound with a fourth compound capable of binding with or attaching to said second component of said second compound making up said third compound, said first component of said second compound being selected from group A consisting of an amino acid, a peptide, or a protein or from group B consisting of a sugar, oligosaccharide or polysaccharide or from group C consisting of a purine, pyrimidine, nucleoside, nucleotide, oligonucleotide or polynucleotide, said second component of said second compound being a sugar, oligosaccharide or polysaccharide and said fourth compound is selected from the group consisting of a peptide or polypeptide, a lectin, or an antibody.

256. A nucleotide in accordance with item 1 wherein said Sig chemical moiety covalently attached to the base B of said nucleotide includes a coenzyme.

257. A nucleotide in accordance with item 256 wherein said coenzyme is selected from the group consisting of thiamine pyrophosphate, flavine mononucleotide, flavine adenine dinucleotide, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide, phosphate, coenzyme A, pyridoxyl phosphate, biotin, tetrahydrofolic acid, coenzyme B_{12}, lipoic acid and ascorbic acid.

258. A method of detecting a first compound which includes in its make-up a nucleotide in accordance with item 256 which comprises contacting said first compound with an apoenzyme corresponding to said coenzyme.

259. A method in accordance with item 258 wherein said coenzyme is flavine adenine dinucleotide (FAD) and wherein said apoenzyme is flavine adenine dinucleotide reductase.

260. A ribonucleotide in accordance with item 101 wherein said Sig chemical moiety includes a coenzyme.

261. A nucleotide in accordance with item 141 wherein said Sig chemical moiety includes a coenzyme.

262. A nucleotide in accordance with item 142 wherein said Sig chemical moiety includes a coenzyme.

263. A nucleotide in accordance with item 143 wherein said Sig chemical moiety includes a coenzyme.

264. A method of detecting a first compound which includes in its make-up a nucleotide in accordance with item 263 which comprises contacting said first compound with an apoenzyme corresponding to said coenzyme.

265. An amino acid or polypeptide in accordance with item 198, wherein said Sig moiety attached to said amino acid or polypeptide includes a coenzyme.

266. An amino acid or polypeptide in accordance with item 265, wherein said coenzyme is selected from the group consisting of thiamine pyrophosphate, flavine mononucleotide, flavine adenine dinucleotide, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, coenzyme A, pyridoxyl phosphate, biotin, tetrahydrofolic acid, coenzyme B_{12}, lipoic acid and ascorbic acid.

267. A method of detecting a first compound which includes in its make-up an amino acid or polypeptide in accordance with item 265, which comprises contacting said first compound with an apoenzyme corresponding to said coenzyme.

268. A method in accordance with item 267, wherein said coenzyme is flavin adenine dinucleotide (FAD) and wherein said apoenzyme is flavine adenine dinucleotide reductase.

269. A monosaccharide or polysaccharide in accordance with item 200, wherein said Sig moiety attached to said monosaccharide or said polysaccharide includes a coenzyme.

270. A monosaccharide or polysaccharide in accordance with item 269, wherein said coenzyme is selected from a group consisting of thiamine pyrophosphate, flavine mononucleotide, flavine adenine dinucleotide, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, coenzyme A, pyridoxyl phosphate, biotin, tetrahydrofolic acid, coenzyme B_{12}, lipoic acid and ascorbic acid.

271. A method of detecting a first compound which includes in its make-up a monosaccharide or polysaccharide in accordance with item 269, which comprises contacting said first compound with an apoenzyme corresponding to said coenzyme.

272. A method in accordance with item 271, wherein said coenzyme is flavine adenine dinucleotide (FAD) and wherein said apoenzyme is flavine adenine dinucleotide reductase.

273. A nucleotide in accordance with item 1, wherein said Sig component comprises a radioactive component.

274. A nucleotide in accordance with item 273, wherein said radioactive component comprises radioactive labeled avidin attached thereto.

275. A nucleotide in accordance with item 273, wherein said Sig component comprises radioactive labeled streptoavidin attached to biotin.

276. A single-stranded polynucleotide comprising one or more nucleotides in accordance with item 1, wherein said Sig component is radioactive.

277. A double-stranded polynucleotide comprising one or more nucleotides in accordance with item 1 and wherein said Sig component thereof is radioactive.

278. A nucleotide in accordance with item 101, wherein said Sig component comprises a radioactive moiety.

279. A polynucleotide comprising a ribonucleotide in accordance with item 101 wherein said Sig moiety is radioactively labeled.

280. A nucleotide in accordance with item 141, wherein said Sig moiety thereof is radioactively labeled.

281. A polynucleotide comprising one or more nucleotides in accordance with item 141 wherein said Sig moiety thereof is radioactively labeled.

282. A nucleotide in accordance with item 142, wherein said Sig moiety thereof is radioactively labeled.

283. A polynucleotide comprising one or more nucleotides in accordance with item 142 wherein said Sig moiety thereof is radioactively labeled.

284. A nucleotide in accordance with item 143, wherein said Sig moiety is radioactively labeled.

285. A polynucleotode comprising one or more nucleotides in accordance with item 143 wherein said Sig moiety thereof is radioactively labeled.

286. A single-stranded polynucleotide comprising one or more nucleotides in accordance with item 143 wherein said Sig component thereof is radioactively labeled.

287. A single-stranded polydeoxyribonucleotide in accordance with item 148, wherein said Sig component thereof is radioactively labeled.

288. A polynucleotide containing one or more nucleotides in accordance with item 1, or item 101, or item 141, or item 142, or item 143 wherein said Sig moiety is radioactively labeled.

289. A polynucleotide containing one or more nucleotides in accordance with item 1 or item 101, or item 141, or item 142, or item 143, wherein said Sig chemical moiety comprises biotin attached to radioactively labeled avidin.

290. A polynucleotide containing one or more nucleotides in accordance with item 1 or item 101, or item 141, or item 142, or item 143, wherein said Sig chemical moiety comprises biotin attached to radioactively

291. A polynucleotide containing one or more nucleotides in accordance with item 1, or item 101, or item 141, or item 142, or item 143, wherein said Sig chemical moiety comprises biotin and radioactively labeled avidin or strepavidin attached to said biotin.

292. A method of determining the presence of a polynucleotide, comprising one or more nucleotides in accordance with item 1, or item 101, or item 141, or item 142, or item 143, wherein said Sig component of said nucleotide comprises biotin which comprises bringing said polynucleotide into contact with radioactive labeled avidin to bind said biotin to said radioactive labeled avidin and determining the presence of the biotin bound to said radioactive labeled avidin by detecting the radioactivity of the biotin-bound radioactive avidin.

293. A method of determining the presence of a polynucleotide, comprising one or more nucleotides in accordance with item 1, or item 101, or item 141, or item 142, or item 143, wherein said Sig component of said nucleotide comprises biotin which comprises bringing said polynucleotide into contact with radioactive labeled strepavidin to bind said biotin to said radioactive labeled strepavidin and determining the presence of the biotin bound to said radioactive labeled strepavidin by detecting the radioactivity of the biotin-bound radioactive strepavidin.

294. A polynucleotide containing one or more nucleotides in accordance with item 1 or item 101, or item 141, or item 142, or item 143, wherein said Sig moiety comprises a radioactively labeled antibody.

295. A polynucleotide containing one or more nucleotides in accordance with item 1 or item 101, or item 141, or item 142, or item 143, wherein said Sig moiety comprises a radioactively labeled protein.

296. A polynucleotide containing one or more nucleotides in accordance with item 1 or item 101, or item 141, or item 142, or item 143, wherein said Sign moiety comprises a radioactively labeled lectin.

The following examples are illustrative of various embodiments of the practices of this invention:

EXAMPLE I

Biotinyl-N-hydroxysuccinide ester (BNHS) was prepared according to a method of Becker et al, P.N.A.S. 68 2604 (1971). Biotin (0.24 g, 1.0 mmol) was dissolved in 5ml dry dimethylformamide. Dicyclohexylcarbodimide (0.21 g, 1.0 mmol) and N-hydroxysuccinimide (.12 g, 1.0 mmol) were added and the solution stirred at room temperature for 15 hours. After filtration of the subsequent precipitate, the filtrate was evaporated at reduced pressure the residue was washed twice with ethanol and reccovered from hot isopropyl alcohol to yield a white crystalline product having a m.p. of 216-218°C.

EXAMPLE II

Biotinyl-1,6-diaminohexane amide was prepared as follows: A solution of 1,6-diaminohexane (320 mg, 2.0 mmol), dissolved in 50 ml water, was brought to pH 8.5 by addition of carbon dioxide. Biotinyl-N-hydroxysuccinimide ester (100 mg, 0.29 mmol), dissolved in 10 ml dimethylformamide, was added. After 18 hours at room temperature the mixture was evaporated and the residue washed with ether and subsequently dried in a dessicator.

EXAMPLE III

Polybiotinylated poly-L-lysine was prepared by the following procedure. Polylysine (100 umol lysine) dissolved in 2 ml 0.1 M sodium borate, pH 8.5 was added to biotinyl-N-hydroxysuccimide ester (17.5 mg, 50 umol) dissolved in 0.5 ml dimethylformamide. After stirring at room temperature for 18 hours, the mixture was dialyzed against 10 mM tris buffer, pH 7.5.

EXAMPLE IV

Oligodeoxyribonucleotides were end-labeled using cytidine-5'-triphosphate and terminal transferase as follows. Purified phage DNA, alkali sheared with 0.2 N sodium hydroxide and diluted to 2 A_{260} units/ml in potassium cacodylate (0.1 M), tris base (25 mm), cobalt chloride (1 mM) and dithiothreitol (0.2 M) were used. To this DNA solution (1 ml) was added cytidine-5'-triphosphate (10 mmol) and terminal transferase (200 units). After incubating at 37° for 5 to 8 hours the reaction was stopped by the addition of neutralized phenol (100 ul), 0.5 M EDTA (100 ul) and 1% sodium dodecyl sulfate (100 ul). The DNA was purified by gel filtration chromatography through Sephadex G-100 followed by precipitation with ethanol.

EXAMPLE V

Biotin and polybiotinylated poly-L-lysine were coupled to oligoribonucleotides using a carbodimide coupling procedure described by Halloran and Parker. J. Immunol., 96 373 (1966). As an example, DNA (1 ug/ml), 1 ml) in tris buffer pH 8.2, sheared with 0.1 N sodium hydroxide was denatured by boiling for 10 minutes and quick cooling in an ice bath. Biotinyl-1,6-diaminohexane amide (2 mg, 6 umol) or polybiotinylated poly-L-lysine (2 mg) and 1-ethyl-3-diisopropylaminocarbomide HCl (10 mg, 64 umol) were added, and the pH readjusted to 8.2. After 24 hours at room temperature in the dark, the mixture was dialyzed against 10 mM tris buffered saline. DNA was precipitated ethanol.

EXAMPLE VI

Biotin, conjugated to cytochrome C, was prepared by the following procedure. To a solution of cytochrome C (10 mg) in 1 ml of 0.1 M sodium borate, pH 8.5 was added biotinyl-N-hydroxysuccinimide ester (10 mg, 29 umol) in 1 ml dimethyl formamide. After 4 hours at room temperature, the biotinylated protein was purified by gel filtration chromatography through a Sephadex G-50 column.

EXAMPLE VII

Formaldehyde coupling of cytochrome C-biotin and polybiotinylated poly-L-lysine to oligodeoxyribonucleotides were carried out using a method similar to that described by Manning et al. Chromosoma, 53, 107 (1975). Oligodeoxyribonucleotide fragments obtained by sodium hydroxide shearing of purified DNA (100 ug/ml in 10 mM triethanolamine, pH 7.8 were denatured by boiling for 10 minutes followed by quick cooling in ice. Cytochrome C-biotin 0.05 g ml or polybiotinylated poly-L-lysine solution (0.05 ml) dissolved 3 mg/ml in 10 mM triethanolamine, pH 7.8 was added to 1 ml at the denatured oligodeoxyribonucleotide solution along with 0.1 ml of 6% formaldehyde in 10 mM triethanolamine, pH 7.8. After stirring at 40° for 30 minutes the mixture was dialyzed against the same buffer. The oligodeoxyribonucleotide-biotin complex was finally purified by gel filtration chromatography on Sephadex G-100 followed by precipitation from ethanol.

EXAMPLE VIII

Double stranded polydeoxyadenylic acid:polybiotinylated deoxyuridylic acid was synthesized as follows. The double stranded oligonucleotide polydeoxyadenylic acid:polythymidylic acid (20 ug) of length 300 basic pairs, dissolved in 200 ul exonuclease III buffer consisting of Tris-HCl pH 8.0 (70 mM); magnesium chloride (1.0 mM) and dithiothreitol (10 mM) was incubated with 100 units exonuclease III for 20 minutes at 20°C. The partially digested oligonucleotide was immediately extracted with phenol, and the DNA was precipitated with 70% aqueous ethanol. The partially digested oligonucleotide was redissolved in 20 ul 5mM tris-HCl pH 7.6 and incubated at 20°C, for 2 hours in a reaction containing 2'--deoxy-adenosine-5'-triphosphate (15 uM) thymidine-5'-triphosphate (the amount determines the degree of substitution) and biotinylated 5-(3-amino-1-propene) 2'-deoxyuridine-5'-triphosphate (5 uM), Klenow DNA polymerase I (200 units) dissolved in 0.1 mM) potassium phosphate, pH 8.0 at a concentration of 0.2 units/ul. The biotinylated poly dA:poly dT, biotinyl dU was purified by gel filtration chromatography on Sephadex G-100. The DNA was enthanol

precipitated and redissolved in 20 ul of solution containing sodium acetate pH 4.6 (30 mM), sodium chloride (50 mol), zinc sulfate (1 mM) and glycerol (5%). S. nuclease (200 units) was added, and the reaction was incubated at 37° for 10 minutes. The reaction was stopped with 1 ml ammonium acetate (4 M) and 6 ml ethanol. The DNA was repurified by G-100 gel filtration chromatography and ethanol precipitation.

EXAMPLE IX

Ligation of poly dA:poly dT, biotinyl dU to oligodeoxyribonucleotides was accomplished as follows: DNA fragments from alkali sheared purified DNA (as described in Example VIII) were digested with S. nuclease and repurified by phenol extraction and ethanol precipitation. Blunt ended DNA fragments (1 ug) and poly dA:poly dT, biotinyl dU (2 ug) were dissolved in 6 ul at a buffer containing tris-HCl pH 7.4 (66 mM), magnesium chloride (6.6 mM), adenosine triphosphate (24 mM) and dithiothreitol (1.0mM), T_4 DNA ligase (50 units) was added, and the volume brought to 20 ul with water. The reaction was incubated 3 hours at 37°C. The DNA was purified by gel filtration chromatography through Sephadex G-100 and was ethanol precipitated.

EXAMPLE X

5-Hydroxymethyl-2'-deoxycytidylic acid was prepared by enzymatic hydrolysis of non glycosylated phage T_4 DNA. Purified phage DNA (2 mg), dissolved in 1 ml 50 mM tris pH 7.4 and 10 mM magnesium chloride, was incubated 20 hours with deoxyribonuclease I at 37°. The pH was adjusted to 9.0 and sodium chloride (20 mM) added. Snake venom phosphodiesterase (0.05 g units in 0.5 ml water) was added and incubation continued at 37° for 5 hours. An additional 0.05 units phosphodiesterase was added and incubation continued 18 hours. Nucleotides were separated by gel filtration chromatography through Sephadex G-50. 5-hydroxymethyl-2'-deoxycytidylic acid was purified by reverse phase high pressure liquid chromatography.

EXAMPLE XI

5-(4-aminobutylaminomethyl)-2'-deoxyuridylic acid was obtained by enzymatic hydrolysis of DNA from phage ØW-14. The phage was grown on Pseudomonas acidovorans 29 according to Kropinski and Warren. Gen. Virol. 6, 85 (1970), and the phage DNA purified according to Kropinski et al. Biochem. 12, 151 (1973). The DNA was enzymatically hydrolyzed with deoxyribonuclease I and snake venom phosphodiesterase using the procedure described elsewhere (Example X). 5-(4-aminobutylaminomethyl)-2'-deoxyuridylic acid was purified by reverse phase high-pressure liquid chromatography.

EXAMPLE XII

Biotinylated-5-(4-aminobutylaminomethyl)-2'-deoxyuridylic acid was prepared as follows: Biotinyl-n-hydroxysuccinimide ester (70 mg 0.2 m mol) dissolved in 1 ml dimethylformamide was added to 5-(4-aminobutyl-aminomethyl)-2'-deoxyuridylic acid in 20 ml 0.1 M sodium borate pH 8.5. After 4 hours the solution was concentrated to 0.5 ml by evaporation, and the biotinylated nucleotide was purified by reverse phase high pressure liquid chromatography.

EXAMPLE XIII

5-formyl-2'-deoxyuridine prepared according to Mertes and Shipchandler, J. Heterocyclic Chem. 1, 751 (1970). 5-hydroxymethyluricil (1 mmol) dissolved in 20 ml dimethylsulfonate was heated at 100°C. with manganese dioxide (2.5 mmol) for 15 minutes. The solvent was evaporated at reduced pressure. The residue was taken up in hot ethanol and recrystallized from ethanol to yield 5-formyluracil. 5-formyluracil (0.10 g) was silylated and dissolved in dry acetonitrile (2.5 ml), 2-deoxy-3,5-di-0-p-toluyl-D-ribofuranosyl chloride (Bhat, Syn. Proc. in Nucleic Acid Chem., Vol. I, p. 521 (1968) (0.22 g) and molecular sieves (0.2 g) were added, and the mixture stirred at 25°C. for 40 hours under anhydrous conditions. The mixture was

filtered and evaporated. The resulting oil was treated with anhydrous ethanol (2 ml) and chromatographed on silica gel to obtain the partially pure anomer which was recrystallized from ethanol (M.P. 195-196°C.) The toluyl groups were removed by reaction of the product in methanol benzene with sodium methoxide. The mixture was neutralized with Dowex 50 (H^+). 5-formyl-2'-deoxyuridine was recrystallized from ethanol M.P. 175-176°C.

EXAMPLE XIV

Biotin was coupled to 5-formyl-2'-deoxyuridine as follows: To 5-formyl-2'-deoxyuridine (.320 g, 1.0 mmol) dissolved in 300 ml 0.05 M sodium borate, was added biotinyl-1,6-diaminohexane amide (0.74 g, 2 mmol). After stirring one hour, sodium borohydride (0.2 g, 5 mmol) was added and stirring continued for an additional 4 hours followed by the addition of 8 ml 1M formic acid. The biotinated compound was purified by reverse phage HPLC eluting with methanol:0.5 M triethyl ammonium acetate, pH 4.0.

EXAMPLE XV

Biotin was coupled to 5-amino-2'-deoxyuridine as follows: 5-amino-2'-deoxyuridine (0.24 g, 1 mmol), biotin (0.25 g, 1 mmol) and dicyclohexylcarboimide (0.21 g, 1 mmol) were dissolved in dry dimethyl formamide and stirred at room temperature overnight. After filtration and evaporation of the solvent, the residue was washed with ether. The biotin-coupled product was purified by reverse phase high pressure liquid chromatography using a water methanol gradient.

EXAMPLE XVI

5-(oxy)acetic acid-2'-deoxyuridine was prepared according to a procedure of Deschamps and Declerq, J. Med. Chem., 21, 228 (1978). 5-hydroxy-2-deoxyuridine (282 mg, 1.15 mmol) was dissolved in 1.16 ml, 1N potassium hydroxide (1.16 mmol) after which iodoacetic acid (603 mg, 3.4 mmol) in 1 ml water was added. After reaction at room temperature for 48 hours 1N HCl (1.06 ml) was added. Concentration of this solution and addition of ethanol yielded a precipitate which was filtered, washed with cold ethanol and recrystallized from hot ethanol.

EXAMPLE XVII

Biotinyl-1,6-diaminohexane amide was coupled to 5-(oxy)acetic acid-2'-deoxyuridine as follows: Biotinyl-1,5-diaminohexane amide (0.74 g, 0.2 mmol), 5-(oxy)acetic acid-2'-deoxyuridine (0.60 g, 0.2 mmol) and dicyclohexylcarboimide (0.41 g, 0.2 mmol) were dissolved in 5 ml dry dimethylformamide and remained overnight at room temperature. The reaction was subsequently filtered and the solvent removed by evaporation. The residue was washed with .1N HCl and ether. The biotinated uridine derivative was purified by reverse phase high pressure liquid chromatography using a water-methanol gradient.

EXAMPLE XVIII

Phosphorylation of 5-substituted pyrimidine nucleosides was accomplished by the general procedure described below for biotinated-5-(oxy)acetic acid-2'-deoxyuridine. The nucleotide (0.16 g, 0.5 mmol) was dried by repeated evaporation from dry pyridine and redissolved in 10 ml dry pyridine. Monomethoxytrityl chloride (0.30 g, 0.8 mmol) was added and the mixture stirred at room temperature in the dark for 18 hours. The solution was diluted with chloroform (200 ml) and extracted with 0.1 M sodium bicarbonate. The organic layer was dried and evaporated. The tritylated nucleoside was redissolved in dry pyridine (20 ml) and acetylated by reaction at room temperature with acetic anhydride (0.1 ml, 20 mmol). The mixture was cooled to 4°C. and methanol (40 ml) added. After stirring 10 hours at room temperature, the reaction was concentrated by evaporation. The compound was detritylated by dissolving in 1% benzene sulfonic acid in chloroform (20 ml). After evaporation of solvent the nucleoside was purified by chromatography on silica gel eluting with 2% methanol:chloroform. The 3'-acetylated nucleoside was dried by repeated evaporation of

dry pyridine. A mixture of phosphorous oxychloride (100 ul. 1 mmol), (1-H), 1,2.4-triazoic (140 mg. 2.2 mmol) and triethylamine (260 ul, 2.0 mmol) was stirred in 5 ml anhydrous dioxane at 10°-15°C. for 30 minutes and at room temperature for 1 hour. This was added to the 3'-acetylated nucleoside, and the mixture stirred at room temperature for 1 hour after which it was cooled to 0°C. Water (5 ml) was added and the reaction stirred at room temperature for 18 hours. Barium chloride (100 mg, 5 mmol) was added and the barium salt of the nucleotide collected by filtration. The salt was washed with water and ether. The barium salt was converted to the sodium salt by stirring with Dowex 50 (Na^+ form) in 10 ml water for 4 hours at room temperature. 2 N sodium hydroxide (2N. 10 ml) was added and the reaction stirred for 15 minutes at room temperature after which it was neutralized by addition of excess Dowex 50 (H^+) form. The deacetylated nucleotide was concentrated by evaporation and purified by reverse phase high-pressure chromatography.

EXAMPLE XIX

5-substituted pyrimidine triphosphates were chemically prepared from their respective 5' monophosphates using a procedure of Michelson. Biochem Biophys Acta, 91, 1, (1964). The example of 5-hydroxymethyl-2'-deoxycytidine-5'-triphosphate will be given. The others were similarly prepared. 5-hydroxymethyl-2' deoxycytidylic acid (free acid) (0.63 g, 0.2 mmol) was converted to its tri-n-octylammonium salt by suspending in methanol and addition of tri-n-octylammonium hydroxide (0.74 g, 0.2 mmol). The suspension was refluxed until a clear solution was obtained and the solvent removed under vacuum. The salt was dried by dissolution in and subsequent evaporation from dry pyridine several times. To the salt, dissolved in dry dimethylformamide (0.1 ml) and dioxane (1 ml) was added diphenylphosphochloridate (0.1 ml) and tri-n-butylamine (0.2 ml). After 25 hours at room temperature, solvent was removed and ether was added to precipitate the nucleoside-5'-diphenylpyrophosphate. This was dissolved in dioxane (0.5 ml) and a solution of di(tri-n-butylammonium) pyrophosphate (0.5 mmol) in 1 ml pyridine was added. After 45 minutes at room temperature, the mixture was conentrated under vacuum to a small volume. The crude product was precipitated with ether. This was dissolved in 0.1 M phosphate buffer pH 8.0. The trisphosphate was purified by chromatography on DEAE cellulose eluting with a gradient of 0.1 to 0.6 M triethylammonium bicarbonate ph 7.5.

EXAMPLE XX

DNA was labeled with 5-substituted pyrimidine triphosphates by nick translating DNA in the presence of the appropriate triphosphate. An example follows for labeling purified DNA with biotinylated 5-formyl-2'-deoxyuridine. DNA (20 ug/ml) was incubated at 14°C. in the presence of magnesium chloride (5 mM) 2'-deoxycytidine-5'-triphosphate (15 mM), 2'deoxyadenosine-5'-triphosphate (15 uM), 2'-deoxyguanosine-5'-triphsophate (15 uM), biotinylated-5-formyl-2'-deoxyuridine-5'-triphosphase (20 uM), activated pancreatic deoxyribonuclease I (13 mg/ml), E. coli deoxyribonuclease acid, polymerase I (40 units/ml) and tris HCL, pH 7.4 (50 mM). After 2 hours the reaction was stopped by addition of 0.3 M EDTA (.05 ml) followed by heating at 65° for 5 minutes. Labeled oligonucleotide was purified by gel filtration chromatography through Sephadex G-100 and precipitation from cold ethanol.

EXAMPLE XXI

PRECIPITATION OF GLUCOSYLATED DNA BY CONCANAVALIN A

Reaction mixtures (1.0 ml) were prepared in 1.5 ml eppendorf tubes as follows:

Sodium potassium phosphate, pH 6.5	10mM
NaCl	150mM
$MgSO_4$	5mM
$CaCl_2$	1mM
DNA (T4 of calf thymus)	50ug
Cancanavalin A (10 mg/ml)	50-500 ug

Reactions were started by the addition of concanavalin A (Con A). The solutions were mixed and left at room temperature for 60 minutes. The tubes were centrifuged at 1200 g for 15-20 minutes. The supernatants were diluted and the A_{280} was measured.

Since Con A absorbs at 260 nanometers, control solutions lacking DNA but containing Con A were prepared. The Con A absorbance was substracted from the absorbence obtained in the complete reaction mixtures.

The results of this reaction are presented in accompanying Figure 1.

EXAMPLE XXII

BINDING OF GLUCOSYLATED DNA TO CONCANAVALIN A

Phage T4 DNA and phage DNA were labeled by incorporation of H^3-deoxyadenosine triphosphate into the DNA by nick translation according to the Rigby et al procedure. T4 DNA was nick translated to a specific activity of 5×10^5 cpm/microgram and an average double-standed size of 5 kilobases. Lambda DNA was nick translation to a specific activity of 3×10^5 cpm/microgram and an average double stranded size of 6.0 kilobases as determined by agarose gel electrophoresis. Unincorporated nucleotides were removed from the reaction mixtures by Bio-Gel P-60 chromatography.

Con A sepharose was prepared as described by the manufacturer (Pharmacia). One ml of settled gel contained 18 mg of bound Con A. One ml columns were prepared in sterile pasteur pippetes and were equilibrated with PBS (0.15 M NaCl; .01 M sodium potassium phosphate, pH 6.5).

H^3-DNA samples were prepared in 0.5 ml of buffer (as described in Example XXI but without Con A). T4 DNA solutions contained 176.000 cpm/0.5 ml. and DNA solutions contained 108.000 cp./0.5 ml. A 0.5 ml sample was applied to the column.

A 10.5 ml volume of buffer was passed through the column, and the eluate fractions (0.33 m) were collected and counted in a Beckman LSC-100 scintillation counter in a 3.5 ml reafluor cocktail (Beckman). The results (Figures 2A and 2B) show that non-glucosylated DNA was not bound whereas glucosylated T4 DNA was bound to the column. The bound T4 DNA was removed by washing the column with a higher pH buffer (Tris-HCl, pH 7.2 - 8.2).

Furthermore, consistent with the interaction of glucose and Con A, mannose, when included in the buffer in which the DNA is applied to the column, prevents binding of glucosylated DNA to Con A sepharose. Also, mannose-containing buffer (PBS-containing 0.056 M mannose) removes bound T4 DNA from Con A sepharose (Figures 3A and 3B).

Further illustrative of the practices of this invention directed to nonradioactive methods or techniques of assaying for specific nucleic acids, the following example deals with the use of the sugar-lectin system. This example deals with the use of DNA which is not glycosylated in nature but rather has had a maltotriose group added thereto by way of nick translation described herein. The maltotriose modified dUTP and DNA modified therewith bind specifically to a column of concanvalin A covalently bound to sepharose. By this technique and in accordance with the practices of this invention, there is provided a means for specifically labeling any nucleic acid with sugars. As previously indicated herein, nick translation is only one of a number of techniques and approaches possible for the production of the modified nucleic acids in accordance with this invention.

EXAMPLE XXIII

Lambda DNA was nick translated as described herein with maltotriose coupled to 5-(3-amino-1-propenyl)-2'-deoxyuridine-5' triphosphate and ^{3}H-2'-deoxyadenosine-5'-triphosphate. Under these conditions DNA was substituted to 40 per cent of its thymidine residues with the maltotriose nucleotide and had a specific activity of 8×10^5 counts per minute (cpm) per microgram of DNA. A control sample of DNA substituted only with ^{3}H-dATP had a specific activity of 6×10^5 cpm per microgram DNA. The nick translated DNA samples were purified free of reaction mixture components by Biogel P-60 chromatography as described herein.

The purified samples were applied to Con A-sepharose columns as described in Figures 2A and 2B. The maltotriose-labeled DNA was retained on the column when washed with PBS but was removed by subsequent elution with 10mM Tris-HCl, pH 8.2 (Figure 4A). The unsubstituted tritiated DNA did not bind to the column at pH 7.4 (Figure 4B).

EXAMPLE XXIV

Potentially immunogenic heptenes may be introduced at the 5 position of uridine by a variety of methods in the literature. 5-(perfluorobutyl)-2'-deoxyuridine was synthesized using a method of Cech et al. Nucl. Acids Res. 2, 2183 (1979). Copper-bronze was prepared by reacting copper sulfite (5 g, 20 mmol) with zinc powder (2 g) in 20 ml water. The mixture was decanted, and the residue washed with water and then 5% hydrochloric acid and water. Just before use, the solid (2 g) was activated with 2% iodine in acetone (20 ml). After filtration the residue was washed with acetone:concentrated hydrochloric acid and then pure acetone. Activated copper-bronze (130 mg, 2 mmol) and 1-iodo-1',2,2',3,3',4,4'heptafluorobutane (1.3 mg, 4 mmol) were stirred in 3 ml dimethylsulfoxide at 110°C. for 1 hour. After cooling and filtration, 2'-deoxyuridine (245 mg, 1mmol) was added, and the mixture heated at 110°C. for 1 hour. Water (5 ml) was added, and the mixture extracted with ether. The ether extracts were dried and evaporated under reduced pressure. The residue was chromatographed on a silica gel column eluting with ethylacetate.

EXAMPLE XXV

Tubericydin was substituted at the 5 position by derivitizing the 5-cyano compound, toyocamycin. An example is the synthesis of 4-amino-5 (tetrazol)-5-yl)-7-(B- D -ribofuranosyl) pyrrolo [2,3-d]pyrimidine using a procedure of Schram and Townsend. J. Carbohydrate. Nucleosides:Nucleotides 1, 38 (1974). Toyocamycin (1.0 g) dissolved in water (100 ml) and glacial acetic acid (13 ml) was heated to reflux. Sodium azide (7.5 g) was added in 1.25 g portions over 10 hours. The solution was cooled to 5°C. and the precipitated product collected M.P. 276-277°C.

EXAMPLE XXVI

5-Cyano-2'-deoxyuridine was prepared according to Bleckley et al, Nucl. Acids Res. 2, 683 (1975). 5-Iodo-2'-deoxyuridine (1.0 g, 2.82 mmol) was dissolved in refluxing hexamethyldisilizane (HMDS) (10 ml). Excess HMDS was removed at reduced pressure, and the resulting oil was dissolved in dry pyridine (50 ml). Cuprous cyanide (350 mg, 3.8 mmol) was added, and the solution heated at 160°C. for 20 hours. Pyridine was removed at reduced pressure, and the residue extracted into toluene which was subsequently evaporated. The residue was heated in 50% aqueous ethanol at 100° for 2 hours. The product was purified by reverse-phase high pressure liquid chromatography and recrystallized from ethanol. M.P. 161°C.

EXAMPLE XXVII

4-amino-5-amino methylene-7-(β-D-2-deoxyfuranosyl) pyrrolo[2,3-d]pyrimidine dihydrochloride was obtained as follows. 4-amino-5-cyano-7-(β-D-2-deoxyfuranosyl) pyrrolo[2,3-d]pyrimidine (Toyocamycin) (0.2 g) was dissolved in hydrochloric acid (10 ml). 10% palladium on charcoal (0.1 g) was added as the mixture hydrogenated at 40 psi for 5 hours at room temperature. After filtration the water was evaporated at reduced pressure. The residue was triturated with ethanol, and the product recrystallized from 50% ethanol.

EXAMPLE XXVIII

5-amino-2'-deoxyuridine was prepared from 5-bromo-2'-deoxyuridine according to the procedure of Roberts and Visser. J. Am. Chem.. Soc. 14:665-669 (1952). 5-bromo-2'-deoxyuridine (2g, 6.2 mmol) dissolved in liquid ammonia (20 ml) was scaled in a glass tube and heated at 50° for 5 days. The tube was opened, and the ammonia was evaporated. 5-amino-2'-deoxyuridine was recrystallized from 5 ml water and 75 ml hot isopropyl alcohol.

EXAMPLE XXIX

5-(methylamino)-2'-deoxyuridine (0.2 g) was prepared as follows. 5-cyano-2'-deoxyuridine (0.2 g, 0.05 mol) was dissolved in 1 N hydrochloric acid (10 ml). 10% palladium on charcoal (0.1 g) was added, and the mixture hydrogenated at 40 p.s.i. for 10 hours at room temperature. The mixture was filtered and the water evaporated at reduced pressure. The residue was triturated with ether, and the product was recrystallized from 80% ethanol.

EXAMPLE XXX

Maltose triose was oxidized to the corresponding carboxylic acid by the following method. Maltose triose (0.5 g, 0.94 mmol) was dissolved in water (5 ml). Lead carbonate (0.42 g, 1.1 mmol) and bromine (0.17 ml), 3.3 mmol) were added, and the mixture was allowed to react at room temperature for six days after which no reducing sugar remained. The mixture was filtered, and silver carbonate (0.2 g) added. After refiltering, the filtrate was deionized by elution through Dowex 50 (H^+ form). Evaporation of water and drying in the presence of phosphorus pentoxide yielded the desired product.

EXAMPLE XXXI

Maltose triose was coupled to 5-(3-amino-1-propenyl)-2'-deoxyuridine-5'triphosphate by the following procedure. Oxidized maltose triose (190 mg, 0.18 mmol) was dissolved in dimethylformamide (0.8 ml) and cooled to 4°C. Isobutyl chloroformate (25 mg, 0.18 mmol) and tri-n-butylamine (43 ul, 0.38 mmol) were added, and the solution allowed to react at 4°C. for 15 minutes. 5-(3-amino-1-propenyl)-2'-deoxyuridine-5'-triphosphate (9.0 umol), dissolved in dimethyl formamide (1.2 ml) and 0.1 4 sodium borate and cooled to 4°C., was added to the above solution. The mixture was incubated at 4°C. for 1 hour and at room temperature for 18 hours. It was then loaded on a DEAE-cellulose column and eluted with a gradient of 0.1 to 0.6 M triethylammonium bicarbonate, pH 7.5. The product was finally purified by reverse phase high pressure liquid chromatography.

Following are Examples XXXII and XXXIII. Example XXXII is a method of tagging allylamine modified dUTP with a fluorescein substituent. This is an example of creation of a self detecting nucleic acid probe. Example XXXIII is a method of labeling preformed double helical nucleic acids at the N^2 position of guanine and the N^6 position of adenine. Example XXXVII has the detector molecule linked to the probe. Chromosoma 84: 1-18 (1981) and Exp. Cell Res. 128:485-490, disclose end labeling of RNA with rhodamine. However the procedure of this invention is less disruptive and labels internal nucleotides.

EXAMPLE XXXII

Fluorescein was coupled to 5-(3-amino-1-propyl)-2'-deoxyuridine-5'-triphosphate (AA-dUTP) as follows. AA-dUTP (10 umol), dissolved in 2 ml sodium borate buffer (0.1 m), pH 9.0, was added to fluorescein isothiocyanate (10 mg, 25 umol) dissolved in 1 ml dimethylformamide. After four hours at room temperature the mixture was loaded onto a DEAE-cellulose column equilibrated in triethylammonium bicarbonate buffer, pH 7.5. The fluorescein coupled AA-dUTP was purified by elution with a gradient of from 0.1 to 0.6 m triethylammonium bicarbonate, pH 7.5.

EXAMPLE XXXIII

DNA may be modified by reaction with chemical alkylating agents. Lambda DNA was alkylated in N^2 position of guanine and N^6 position of adenine by reacting DNA with aromatic hydrocarbon 7-bromomethylbenz[a]anthracene. 7-bromomethylbenz[a]anthracene was obtained as follows. 7-methyl[a]-anthracene in carbon disulfide solution was cooled in a freezing mixture and treated dropwise with a molar equivalent of bromine. After 30 minutes, the product in suspension was collected, and was washed with dry ether and recrystallized from benzene. The yield was 66% with melting point 190.5-191.5°C.

DNA, purified from phage Lambda, (1.6 mg) was solubilized in 5.0 ml of 20 mM potassium phosphate pH 6.5. To 4.0 ml of DNA solution was added 500 micrograms 7-bromomethylbenz[a]anthracene in dry

acetone. After 30 minutes at 20°, the DNA was precipitated with two volumes of cold ethanol. The precipitate was washed successively with ethanol, acetone and ether to remove any unbound 7-bromomethylbenz[a]anthracene. Enzymatic hydrolysis of the DNA to nucleosides and subsequent chromatography of the products on Sephadex LH-20 columns, indicated that 18% of the adenine and 48% of the guanine in DNA were modified in N^6 and N^2 positions, respectively.

The modified DNA was made single stranded either by (1) heating to 100° for 5 minutes and rapid cooling or (2) incubating with equal volume of 0.1 M NaOH for 10 minutes and then dialyzing the solution for four hours against 1 ml tris-HCl pH 8.0 containing 0.5 ml EDTA to keep the DNA in single-stranded form.

EXAMPLE XXXIV

A DNA probe was ligated to a synthetic DNA composed of repeated sequences of E. coli lac operator DNA. After hybridization to detect antiprobe sequences, the hybridized DNA was detected by reaction with biotinylated lac repressor which was, in turn, detected by an enzyme linked immuno sorbent assay using goat antibiotin IGG to react with the biotin and a second antibody coupled to horse radish peroxidase. The lac polyoperator DNA has been described by Caruthers (Second Annual Congress for Recombinant DNA Research, Los Angeles, 1982), and it was ligated, in a blunt end ligation, using T4 ligase, to an adenovirus DNA probe. In situ hybridization of the polyoperator-labeled probe DNA was carried out as described by Gerhard et al (Proc. Natl. Acad. Sci. USA, 78, 3755 (1981). Biotinylated lac repressor was prepared as described by Manning et al (Chromosoma, 53, 107-117 (1075) and was applied to adenovirus infected cells, fixed to a glass slide, in Binding buffer composed of (0.01 MK Cl, 0.01 M tris (pH 7.6), 0.01 M $MgSO_4$, 10^{-4} MEDTA, 10^{-4} M DTT, 5% DMSO (dimethyl sulfoxide) and 50 ug ml bovine serum albumin by J. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory (1972). The slides were washed in binding buffer to remove unbound biotinylated lac repressor and then assayed for biotin using the horse radish peroxidase-linked double antibody procedure. This procedure could be adapted to create an affinity column where the probe could be bound to immobilized repressor protein and then removed by elution with a specific inducer, for example, isopropylthigalactoside or thiomethylgalactoside. The affinity of the repressor-operator complex is quite high 10^{-11} M. When a specific inducer binds to the repressor the operator-repressor complex collapses.

EXAMPLE XXXV

5-Bromo-2'-deoxyuridine-5'-phosphate was prepared as follows: 2'-Deoxyuridine-5'-phosphate (6.2 g) was suspended in a mixture of 60 ml pyridine and 30 ml acetic acid. Bromine (0.84 ml) was added with stirring in an ice water bath and stirring continued for 20 hours at room temperature. The solution was concentrated by vacuum. After redissolution in a minimum of water a crude product was precipitated by addition of ethanol. The crude product was chromatographed on Dowex 50 (H^+) and eluted with water. The free acid product was precipitated from the concentrated eluent by addition of ethanol.

EXAMPLE XXXVI

Calf intestine alkaline phosphate was biotinylated as follows: The enzyme (1 mg, 7.7 mmol) was chromatographed on a G-50 column eluting with 0.1 M Hepes buffer pH 8.0 containing 0.1 M sodium chloride. The pooled fractions were reacted with N-biotinyl-6-amino-caproic acid-N-hydroxysuccinimide ester (0.675 mg, 0.77 umol) dissolved in 10 ml diemthylformamide at room temperature for 1 hour. Sodium periodate (0.1 M 125 ul) was added and stirring continued for 2 hours. The mixture as dialyzed at 4° overnight in 0.1 M Hepes buffer pH 8.0 with 0.1 M NaCl after which the pH was adjusted to 7.4. Biotin hydrazide (0.1 M, 0.5 ml) dissolved in 0.1 M Hepes buffer pH 7.4 and 0.1 M NaCl was added and the reaction stirred for 30 minutes at room temperature. The pH was adjusted to 8.0 with 0.2 M sodium carbonate and 0.5 ml of freshly prepared 0.1 M sodium borohydride in water was added, the solution was dialyzed against 0.1 M tris buffer pH 8.0 with 0.1 M NaCl.

EXAMPLE XXXVII

6-Cyano-2'-deoxyuridine-5'-phosphate was prepared similarly to a procedure of Veder et al, J. Carbohydr. Nucleosides, Nucleotides, 5, 261 (1978). 5-bromo-2'-deoxyuridine-5'-phosphoric acid (2.0 g, 15 mmol) dried by successive evaporation from pyridine was dissolved in 50 ml dimethylsulfide. Sodium cyanide (490 mg, 10 mmol) was added and the solution stirred at room temperature for 2 days. The solution was diluted with 400 ml water and the pH adjusted to 7.5. It was applied to a DEAE-cellulose column (HCO^-_3 form) washed with 2000 ml 0.02 M triethylammonium bicarbonate to yield the desired product.

EXAMPLE XXXVIII

6-(Methylamino)-2'-deoxyuridine-5'-phosphoric acid was prepared as follows: 6-Cyano-2'-deoxyuridine-5'-phosphoric acid (0.2 g, 60 mmol) was dissolved in 0.1 M hydrochloric acid. After addition of 10% palladium on charcoal (0.1 g). the mixture was hydrogenated at 40 psi for 20 hours at room temperature. The mixture was filtered. neutralized with lithium hydroxide and lyophilized. The product residue was extracted with ethanol.

EXAMPLE XXXIX

Horse radish peroxidase (20 mg) dissolved in 5 ml distilled water was added to 1.0 ml freshly prepared 0.1 M sodium periodate solution. After stirring at room temperature for 20 minutes it was dialyzed overnight at 4°C. against 1 mM sodium acetate pH 4.4. Biotin hydrazide (2.6 mg, $5^{\times}10$-2 mmol) dissolved 2.0, 0.1 M Hepes buffer pH 7.4 with 0.1 M sodium chloride was brought to pH 8.0 with 0.2 M sodium carbonate and 0.5 ml of a freshly prepared 0.1 M sodium borohydride solution in water was added. After 2 hours at 4°C. the protein was purified on a Sephadex G-50 column eluting with 0.1 M Hepes and 0.1 M NaCl.

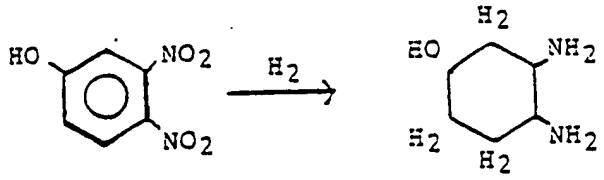
EXAMPLE XL

Carrot acid phosphatase has been mentioned by Brunngraber and Chargaff, J. Biol Chem., (1967) 242, 4834-4840 as a byproduct of the purification of phosphotransferase and has been purified to a specific activity of 460 uM/mg/min at 37°C. with paranitrophenylphosphate as the substrate. The purification involved the steps of (a) absorption of non-specific proteins by DEAE cellulose; (b) acid purification of the enzyme; (c) acetone fractionation; (d) concanvalin A affinity chromatography; (e) hydroxy-apatite chromatography and (f) Sephadex G-100 fractionation. The specific activity of the enzyme subjected to the Sephadex G-100 fractionation due to loss of activity in the preceding affinity chromatography step (d) was 170 uM/mg/m. By changing elution conditions at step (d), these losses can be avoided with the result that the specific activity of the enzyme before the Sephadex G-100 fractionation can be improved to 340 uM/mg/m. The Sephadex G-100 fractionation step should yield an enzyme having a specific activity of 800 uM/mg/m or higher. Carrot acid phosphatase was biotinylated using a procedure of Wilchek et al Biochemistry 6, 247 (1967). To the enzyme (20 mg) dissolved 0.1 M NaCl, pH 5, was added biotin hydrazide (2.0 mg, $7^{\times}10^{-3}$ mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (1 mg, $7^{\times}10^{-3}$ mmol) dissolved in 0.1 M NaCl, pH 5. After 2 hours at 4°C. the enzyme was chromatographed on Sephadex G-50 eluting with 0.1 M sodium acetate, pH 5.0.

Of special importance and significance in the practices of this invention is the utilization of self-signaling or self-indicating or self-detecting nucleic acids, particularly such nucleic acids which are capable of being incorporated in double-stranded DNA and the like. Such self-signaling or self-detecting nucleic acids can be created by covalently attaching to an allylamine substituent making up a modified nucleotide in accordance with this invention a molecule which will chelate specific ions, e.g. heavy metals, rare earths, etc. In general, the chelated ion can be detected either (a) by radioactive emission or (b) by using the ion to catalyze a chromogenic or fluorogenic reaction.

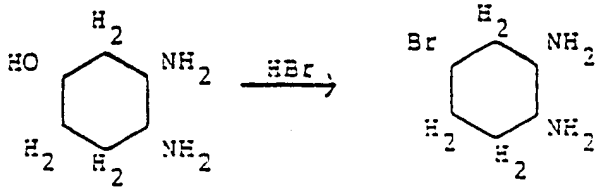
By way of example, a solution of 3,4-dinitro phenol is reduced to 3,4-diamino cyclohexane

1)



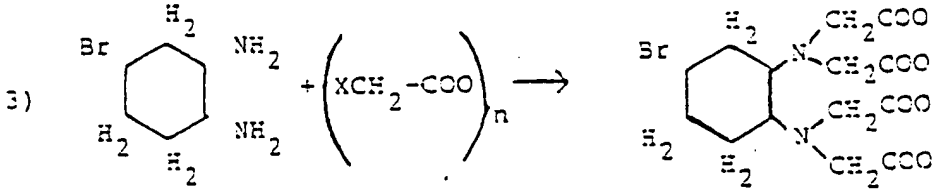
This material is then brominated

2)



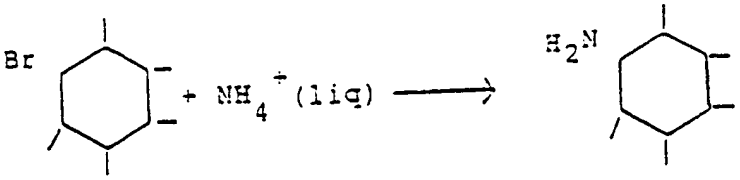
to form 3.4-diamino bromo cyclohexane (dABCH). This compound is reacted with halide (Cl, Br, I) substituted carboxymethyl compound to produce a tetra carboxymethyl derivative or dABCH (TCM-dABCH):

3)



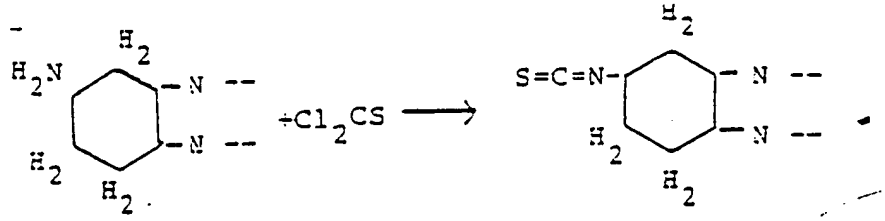
The bromine is substituted by an amino group using soluble ammonia:

4)

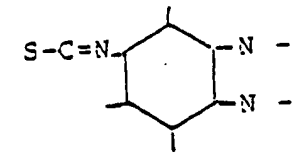


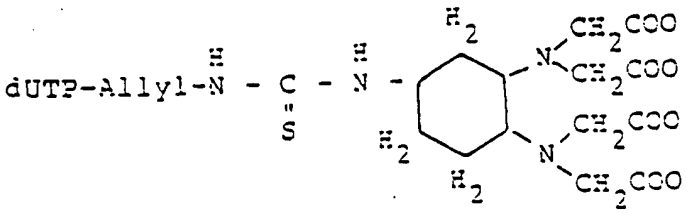
Then this compound is reacted with chloro thiophosgene to produce the isothiocyanate derivative of (TCM-dANCH).

5)



Finally, this compound is reacted with dUTP-allylamine derivative to produce modified dUTP.

6) dUTP-Allylamine +  ⟶



Cobalt or other heavy metal ions or other rare earth ions can be chelated to the compound after step 3 above. Or the nucleic acid can be substituted with this adduct and then the ion added. (Example. cobalt is added at pH 6 where the binding constant is 10^{-9} M).

Cobalt can be assayed by radioactivity. It can also be detected by its ability to oxidize methylene blue to the leuco form in the presence of molecular oxygen. It can be used to oxidze soluble sulfhydro groups to disulfide bonds again in the presence of molecular oxygen.

This type of self-signaling molecule can be used to monitor any nucleic acid hybridization reaction. It is particularly important for detecting nucleic acids in gels (for example, sequencing gels).

With respect to its use in radioactivity, it can be used to tailor the isotope needed, i.e. if a weak or strong β or γ emitter is needed, that isotope can be chelated. Examples of isotopes that can be used are listed immediately hereinafter.

Antimony-124
Antimony-125
Arsenic-74
Barium-133
Barium-140
Beryllium-7
Bismuth-206
Bismuth-207
Cadmium-109
Cadmium-115m
Calcium-15
Carbon-14
Cerium-139
Cerium-141
Cerium-144
Cesium-134
Cesium-137
Chlorine-36
Chromium-51
Cobalt-56
Cobalt-57
Cobalt-58
Cobalt-60
Erbium-169
Europium-152
Gadolinium-153
Gold-195
Gold-199
Hafnium-175
Hafnium-175 + 181
Hafnium-181

Hydrogen-3 see Tritium
Iodine-125
Iodine-131
Iodine-132
Iridium-192
Iron-55
Iron-59
Krypton-85
Lead-210
Lutecium-177
Manganese-54
Mercury-197
Mercury-203
Molybdenum-99
Neodymium-147
Neptunium-237
Nickel-63
Niobium-95
Osmium-185 + 191
Palladium-103
Platinum-195m
Praseodymium-143
Promethium-147
Protactinium-233
Radium-226
Rhenium-186
Rubidium-86
Ruthenium-103
Ruthenium-106
Scandium-44
Scandium-46
Selenium-75
Silver-110m
Silver-111
Sodium-22
Strontium-85
Strontium-89
Strontium-90
Sulphur-35
Tantalum-182
Technetium-99
Tellurium-125m
Tellurium-132
Terbium-160
Thallium-204
Thorium-228
Thorium-232
Thulium-170
Tin-113
Titanium-44
Tritium
Tungsten-185
Vanadium-48
Vanadium-49
Ytterbium-169
Yttrium-88
Yttrium-90
Yttrium-91

Zinc-65
Zirconium-95

Streptavidin, a protein produced by a Streptomyces avidinii is a large molecular weight component of a synergistic pair of compounds which are both present in the culture filtrates of this microorganism. Each of the pair is inactive but in combination are active against gram-negative microorganisms. It has been found that the small component of this antibiotic prevents de novo synthesis of the vitamin biotin and thus, at least in synthetic media, show antimicrobial activity. In complex medium, however, the large component has to be included to exert the same effect on bacteria. This has been shown to be due to the presence of external biotin in the complex medium. The large molecular component has been found to bind external biotin and thus demonstrating the same kind of action as avidin from eggs and oviduct tissues of laying birds.

Streptavidin has been purified and shown to be a 60.000 dalton polypeptide. Like avidin, streptavidin contains four subunits and binds tightly four molecules of biotin. Unlike avidin, however, it is non-glycosylated and it has PI of 5.0 as compared to avidin with PI = 10.5. Due to the difference in pI streptavidin does not have a tendency to non-specifically interact with DNA.

PREPARATION OF STREPTAVIDIN

A semi-synthetic medium containing salt, 1% glucose, 0.1% asparagine, 0.05% yeast extract and trace elements was prepared. The cultures were grown at 26°C. for three days. Mycellium was removed by centrifugation and protein in the supernatant were absorbed to DEAE-cellulose in a batchwise process after pH was adjusted with 1M HCl to 7.2. DEAE-cellulose was filtered off and washed with 20 mM Tris-Hcl (pH 7.2) until no absorbancy at 280 nm was recorded. Streptavidin was eluted with 20 mM Tris-HCl (pH 7.2) containing 0.5 M NaCl. Ammonium sulfate precipitation was used to further concentrate the streptavidin (50% w/v at 4°C.).

The precipitate was dissolved in water and dialyzed against 1.0 M NaCl, 50 mM Na_2CO_3. In the next step affinity column chromatography on iminobiotin sepharose was used. Eluted streptavidin from iminobiotin sepharose column was shown to be chromatographically pure by non-denaturing agarose-gel electrophoresis.

The final purification of streptavidin is accomplished by affinity purification through an iminobiotin-sepharose column. Iminobiotin is an analog of biotin in which the carbonyl of the urea moiety is substituted with an imine function. Iminobiotin will bind avidin and streptavidin at basic pH but the complex is dissociable at acidic pH.

Iminobiotin is prepared from biotin in several steps. Biotin is hydrolyzed by barium hydroxide to cis-3,4-diamino-2-tetrahydrothiophene-valeric acid which is reacted with cyanogen bromide to iminobiotin. The iminobiotin is coupled to amino sepharose via the N-hydroxysuccinimide ester of its hydrobromide salt.

The crude protein mixture from DEAE eluted Streptomyces avidinii incubation media is dissolved in 50 mM sodium carbonate and 1.0 M sodium chloride (pH 11) and applied to an iminobiotin column pre-equilibrated with this solution. The column is eluted at pH 11. Streptavidin is subsequently eluted with 50 mM ammonium acetate, pH 4.0 containing 0.5 M sodium chloride. The eluent is dialyzed three times against 1 mM Tris pH 7.4 and lyophilized to dryness.

In the practices of this invention avidin is useful as a detecting mechanism for labeled DNA, such as biotin-labeled DNA. However, avidin itself, such at about neutral pH, complexes with DNA with the result that any signal derivable from a complex between biotin-labeled DNA and avidin might be lost or be non-detectable in the background due to the complex formation between avidin and unlabeled DNA. This disadvantage of the use of avidin in the practices of this invention is not possessed by streptavidin which does not form a complex with DNA at about neutral pH but is capable of forming a complex with the biotin portion of biotin-labeled DNA.

In another aspect directed to the broad utility of avidin and streptavidin for detecting labeled compounds other than DNA, avidin and streptavidin are particularly effective as detecting mechanisms for labeled proteins, polysaccharides and lipids. By way of example, one can fix to a solid matrix a specific antigen and bind to this antigen an antibody directed against this antigen which itself has been biotinylated. Then one can assay for the presence of this biotinylated antibody by reacting it with avidin or streptavidin complexed with an enzyme, such as calf intestine alkaline phosphatase, or to which fluorescing molecule, as for example fluoroscein has been linked.

The use of the antigen-antibody system for detecting either antigen or antibody is well known. A comparable system is a system based on a glycosylated substrate or molecule and matching or appropriate lectin. In this system the lectin would carry a label, such as fluorescein or appropriate enzyme. In this glycosyl-lectin system the labeled lectin forms a complex with the glycosyl moiety, comparable to the antigen-antibody complex, and this complex comprising the glycosylated molecule and appropriate labeled lectin having the necessary glycosyl or sugar moiety specificity would then present itself eliciting the expected response from the label portion of the labeled lectin making up the glycosyl-lectin complex.

Another aspect of the practices of this invention which is particularly advantageous is to carry out the detection or hybridization in the liquid phase between the DNA sought to be detected and the DNA detecting probe. In this liquid phase system both the DNA molecule to be detected and the appropriate DNA detecting probe are not attached to any insoluble substrate or any insoluble chemical moiety. The advantages of the liquid phase detection system reside in the speed of hybridization or hybrid formation between the DNA to be detected and the appropriate DNA probe therefor. For example, in a solid-liquid system the time required to effect recognition and hybridization formation is about ten times greater than if it were carried out in a completely liquid system, i.e. both DNA to be detected and the detecting DNA are not attached to an insoluble moiety.

The probes prepared in accordance with the practices of this invention are adaptable for use in the detection of viruses and bacteria in fluids as indicated hereinabove. Where the fluids to be examined do not contain large amounts of protein, the viruses therein can be concentrated by absorption on hydroxyapatite and eluted in a small amount of phosphate buffer. When the fluid to be examined contains large amounts of protein, the viruses can be concentrated by high speed centrifugation.

If antibody were available, absorption on an affinity column and elution with acid would be preferable because it would be possible to process many probes in accordance with the practices of this invention at the same time. The bacteria to be examined is usually readily concentrated by centrifugation.

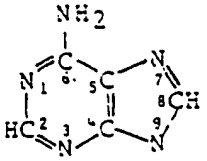
In accordance with the practices of this invention, the identification or characerization of the isolated particles, viruses and bacteria, would be hybridization of the characterizing or identifying DNA thereof with a specific single stranded DNA probe prepared in accordance with the practices of this invention. After hybridization, excess non-hybridized probe DNA would be digested with S. nuclease and exonclease I from E. coli at high salt content to suppress the nicking activity of the S. nuclease, see Vogt, Methods in Enzymology, Vol. 65, pages 248-255 (1980). This nuclease treatment would produce mononucleotides from the excess, non-hybridized single-stranded DNA probe but would leave the double-stranded, hybridized DNA intact. This would then be absorbed at high salt content on Dowex anion exchanger (the nucleotides and the small amount of oligonucleotides will not bind to the resin in high salt concentration). The resulting hybridized DNA would then be identified or characterized by various procedures applicable to the practices of this invention.

The special nucleotides of this invention include a phosphoric acid P moiety, a sugar or monosaccharide S moiety, a base B moiety, a purine or a pyrimidine and a signalling chemical moiety Sig covalently attached thereto, either to the P, S or B moiety. Following are structural formulas of various base B moieties and nucleotides which are modified in accordance with the practices of this invention.

The major purines

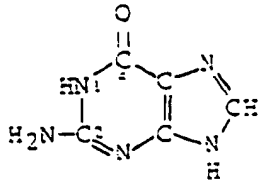
Adenine

(6-aminopurine)



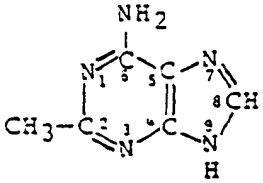
Guanine

(2-amino-6-oxypurine)

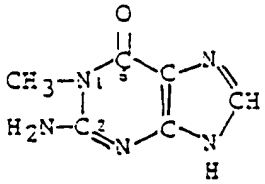


Two minor purines

2-Methyladenine



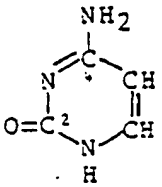
1-Methylguanine



The major pyrimidines

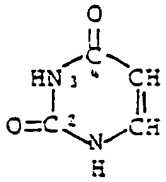
Cytosine

(2-oxy-4-aminopyrimidine)



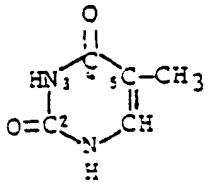
Uracil

(2,4-dioxypyrimidine)



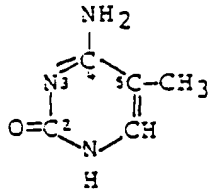
Thymine

(5-methyl-2,4-dioxypyrimidine)

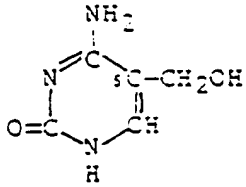


Two minor pyrimidines

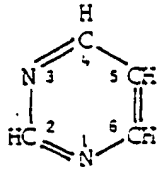
5-Methylcytosine



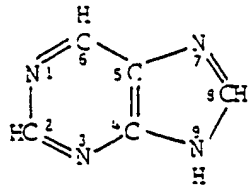
5-Hydroxymethylcytosine



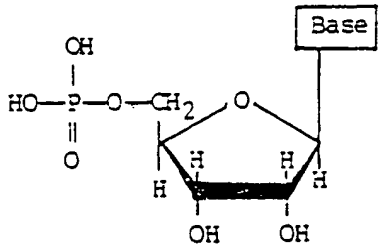
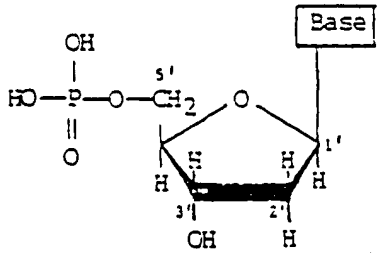
PYRIMIDINE



PURINE



The major ribonucleotides and deoxyribonucleotides.

Ribonucleoside 5'-monophosphates	2'-Deoxyribonucleoside 5'-monophosphates
	
General Structure	General Structure
Names	Names
Adenosine 5'-phosphoric acid (adenylic acid; AMP)	Deoxyadenosine 5'-phosphoric acid (deoxyadenylic acid; dAMP)
Guanosine 5'-phosphoric acid (guanylic acid; GMP)	Deoxyguanosine 5'-phosphoric acid (deoxyguanylic acid; dGMP)
Cytidine 5'-phosphoric acid (cytidylic acid; CMP)	Deoxycytidine 5'-phosphoric acid (deoxycytidylic acid; dCMP)
Uridine 5'-phosphoric acid (uridylic acid; UMP)	Deoxythymidine 5'-phosphoric acid (deoxythymidylic acid; dTMP)

The special nucleotides in accordance with this invention, as indicated hereinabove, in addition to the P, S and B moieties, include a chemical moiety Sig coavalently attached to the P, S and/or B moieties. Of special interest in accordance with the practices of this invention would be those nucleotides having the general formula.

P - S - B - Sig

wherein P is the phosphoric acid moiety including mono-, di-, tri-or tetraphosphate, S the sugar monosaccharide moiety, B the base moiety, either a purine or a pyrimidine. The phosphoric acid moiety P is attached at the 3' and/or the 5' position of the S moiety when the nucleotide is a deoxyribonucleotide and at

the 2', 3' and/or 5' position when the nucleotide is a ribonucleotide. The base B moiety is attached from the N1 position or the N9 position to the 1' position of the S moiety when the base moiety is a pyrimidine or a purine, respectively. The Sig moiety is covalently attached to the B moiety of the nucleotide and when so attached is capable of signalling itself or makes itself self-detecting or its presence known and desirably or preferably permits the incorporation of the resulting nucleotide P - S - B - Sig into or to form a double-stranded helical DNA or RNA or DNA-RNA hybrid and/or to be detectable thereon.

Another special nucleotide in accordance with this invention is characterized by the general formula:

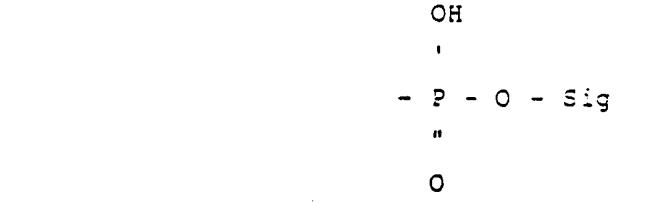
Such nucleotides in accordance with this invention would be characterized as ribonucleotides. The phosphoric acid moiety is attached at the 2', 3' and or 5' position of the sugar S moiety and the base B being attached from the N1 position or the N9 position to the 1' position of the sugar S moiety when said base is a pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the sugar S moiety and said Sig chemical moiety when attached to said S moiety is capable of signalling itself or making itself self-detecting or its presence known and preferably permits the incorporation of the ribonucleotide into its corresponding double-stranded RNA or a DNA-RNA hybrid.

Such nucleotides

desirably have the Sig chemical moiety attached to the C2' position of the S moiety or the C3' position of the S moiety.

Still further, nucleotides in accordance with the practices of this invention include the nucleotides having the formula,

wherein P is the phosphoric acid moiety, S the sugar moiety and B the base moiety. In these special nucleotides the P moiety is attached to the 3' and/or the 5' position of the S moiety when the nucleotide is deoxyribonucleotide and at the 2', 3' and/or 5' position when the nucleotide is a ribonucleotide. The base B is either a purine or a pyrimidine and the B moiety is attached from the N1 or the N9 position to the 1' position of the sugar moiety when said B moiety is a pyrimidine or a purine, respectively. The Sig chemical moiety is covalently attached to the phosphoric acid P moiety via the chemical linkage



said Sig, when attached to said P moiety being capable of signalling itself or making itself self-detecting or its presence known and desirably the nucleotide is capable of being incorporated into a double-stranded polynucleotide, such as DNA, RNA or DNA-RNA hybrid and when so incorporated therein is still self-

detecting.

It is pointed out that the special nucleotides in accordance with the practices of this invention described or defined hereinabove by the general formula P - S - B - Sig, also include nucleotides wherein the Sig chemical moiety is covalently attached to the B moiety at the N^6 or 6-amino group position when the B moiety is adenine or the N^2 or 2-amino group position when the B moiety is guanine or the N^4 or 4-amino group position when the B moiety is cytosine. The resulting nucleotides containing the Sig moiety attached thereto are capable of signalling themselves or making themselves self-detecting or their presence known and being detectable is a double-stranded or DNA, RNA or DNA-RNA hybrid.

By way of summary, as indicated hereinabove with respect to the make-up of the various special nucleotides in accordance with this invention, the special nucleotides can be described as comprising a phosphoric acid moiety P, a sugar moiety S and a base moiety B, a purine or pyrimidine, which combination of P-S-B is well known with respect to and defines nucleotides, both deoxyribonucleotides and ribonucleotides. The nucleotides are then modified in accordance with the practices of this invention by having covalently attached thereto, to the P moiety and/or the S moiety and/or the B moiety, a chemical moiety Sig. The chemical moiety Sig so attached to the nucleotide P-S-B is capable of rendering or making the resulting nucleotide, now comprising P-S-B with the Sig moiety being attached to one or more of the other moieties, self-detecting or signalling itself or capable of making its presence known per se, when incorporated into a polynucleotide, especially a double-stranded polynucleotide, such as a double-stranded DNA, a double-stranded RNA or a double-stranded DNA-RNA hybrid. The Sig moiety desirably should not interfere with the capability of the nucleotide to form a double-stranded polynucleotide containing the special Sig-containing nucleotide in accordance with this invention and, when so incorporated therein, the Sig-containing nucleotide is capable of detection, localization or observation.

The Sig moiety employed in the make-up of the special nucleotides of this invention could comprise an enzyme or enzymic material, such as alkaline phosphatase, glucose oxidase, horseradish peroxidase or ribonuclease. The Sig moiety could also contain a fluorescing component, such as fluorescein or rhodamine or dansyl. If desired, the Sig moiety could include a magnetic component associated or attached thereto, such as a magnetic oxide or magnetic iron oxide, which would make the nucleotide or polynucleotide containing such a magnetic-containing Sig moiety detectable by magnetic means. The Sig moiety might also include an electron dense component, such as ferritin, so as to be available by observation. The Sig moiety could also include a radioactive isotope component, such as radioactive cobalt, making the resulting nucleotide observable by radiation detecting means. The Sig moiety could also include a hapten component or per se be capable of complexing with an antibody specific thereto. Most usefully, the Sig moiety is a polysaccharide or oligosaccharide or monosaccharide, which is capable of complexing with or being attached to a sugar or polysaccharide binding protein, such as a lectin, e.g. Concanavilin A. The Sig component or moiety of the special nucleotides in accordance with this invention could also include a chemiluminescent component.

As indicated in accordance with the practices of this invention, the Sig component could comprise any chemical moiety which is attachable either directly or through a chemical linkage or linker arm to the nucleotide, such as to the base B component therein, or the sugar S component therein, or the phosphoric acid P component thereof.

The Sig component of the nucleotides in accordance with this invention and the nucleotides and polynucleotides incorporating the nucleotides of this invention containing the Sig component are equivalent to and useful for the same purposes as the nucleotides described in EP-A2-0 063 879. More specifically, the chemical moiety A described in EP-A2-0 063 879 is functionally the equivalent of the Sig component or chemical moiety of the special nucleotides of this invention. Accordingly, the Sig component or chemical moiety of nucleotides of this invention can be directly covalently attached to the P, S or B moieties or attached thereto via a chemical linkage or linkage arm as described in EP-A2-0 063 879 as indicated by the dotted line connecting B and A of the nucleotides of EP-A2-0 063 879 The various linker arms or linkages identified in EP-A2-0 063 879 are applicable to and useful in the preparation of the special nucleotides of this invention.

A particularly important and useful aspect of the special nucleotides of this invention is the use of such nucleotides in the preparation of DNA or RNA probes. Such probes would contain a nucleotide sequence substantially matching the DNA or RNA sequence of genetic material to be located and/or identified. The probe would contain one or more of the special nucleotides of this invention. A probe having a desired nucleotide sequence, such as a single-stranded polynucleotide, either DNA or RNA probe, would then be brought into contact with DNA or RNA genetic material to be identified. Upon the localization of the probe and the formation of a double-stranded polynucleotide containing the probe and the matching DNA or RNA material to be identified, the resulting formed double-stranded DNA or RNA-containing material would then

be observable and identified. A probe in accordance with this invention may contain substantially any number of nucleotide units, from about 5 nucleotides up to about 500 or more, as may be required. It would appear that 12 matching, preferably consecutive, nucleotide units would be sufficient to effect an identification of most of the DNA or RNA material to be investigated or identified, if the 12 nucleotide sequence of the probe matches a corresponding cooperative sequence in the DNA or RNA material being investigated or to be identified. As indicated, such probes may contain one or more of the special Sig-containing nucleotides in accordance with this invention, preferably at least about one special nucleotide per 5-10 of the nucleotides in the probe.

As indicated hereinabove, various techniques may be employed in the practices of this invention for the incorporation of the special nucleotides of this invention into DNA and related structures. One particularly useful technique referred to hereinabove involves the utilization of terminal transferase for the addition of biotinated dUMP onto the 3' ends of a polypyrimidine or to single-stranded DNA. The resulting product, such as a single-stranded or cloned DNA, which has biotinated dUMP attached to the 3' ends thereof, can be recovered by means of a Sepharose-avidin column wherein the avidin would complex with the biotinated dUMP at the ends of the DNA and be subsequently recovered. In accordance with the practices of this invention hybridization to mRNA could be accomplished in solution and the resulting hybrid recovered via a Sepharose-avidin column and the mRNA recovered therefrom. Similar techniques could be employed to isolate DNA-RNA hybrids. This technique employing terminal transferase for the addition of the special nucleotides in accordance with this invention is widely applicable and the resulting modified nucleotides containing the special nucleotides in accordance with this invention including the special biotinated nucleotides or the special glycosylated nucleotides could be selectively recovered via complexing with avidin upon a Sepharose-avidin column or complexing with a lectin, such as Concanavalin A or a Sepharose-Concanavalin A column.

Illustrative of the practices of this invention, biotinated dUTP was added to the 3' ends of d pT 4 as well as single and double stranded DNA employing terminal transferase and the resulting product was purified through G-50 Sepharose and separated on a Sepharose-avidin affinity column. It was found that 69 % of the d pT 4 molecules were biotinated and recovered on the Sepharose-avidin column. The results of this experiment established that terminal transferase added biotinated dUMP to the 3' ends of a polypyrimidine.

The detection of nucleic acids to which specific molecules have been covalently attached can be effected through the use of many naturally occurring proteins to which small molecules are known to specifically bind. In this procedure the small molecules are bound to the nucleotide using the allyl amine side chain. These nucleotides are then incorporated into specific nucleic acids using a DNA or RNA polymerase or ligase reaction or a chemical linkage. After annealing this probe with a complementary antiprobe sequence, the presence of the probe is assayed for by the specific binding of the protein to the ligand covalently bound to the probe.

Examples of protein-ligand reactions that are appropriate for this type of detector system include:

1. Enzymes and allosteric effector or modulator molecules. An example of this is the enzyme threonine dehydratase which is a heterotropic enzyme in that the effector molecule, L-isoleucine, is different than the substrate, L-threonine, J. Monod, J. Wyman and J.P. Changeux (1965), J. Mol. Biol. 12:88-118.

2. Effector molecules involved in regulation. An example of this is the specific binding of 3',5-cyclic adenosine monophosphate to the cyclic AMP receptor protein, I. Pastan and R. Perlman, Science 169:339-344 (1969). Another example is the lactose repressor molecule and the inducer molecules isopropyl-thiogalactoside or thiomethylgalactoside. These two inducer molecules are called gratuitous inducers in that they are not metabolized by the enzymes they induce, W. Gilbert and B. Muller-Hill, Proc. Natl. Acad. Sci. (US), 70:3581-3584, (1973).

3. Hormone receptors and other receptors on the surface of the cell to which organic molecules will specifically bind. An example of this is the epinephrine-epinephrine receptor system in which epinephrine is bound in a steriospecific manner with a high affinity to the receptor. With this system, since the receptor protein is insoluble in water, it will be imbedded in a lipid bilayer structure as for instance a liposome. Suitable detector systems would include specific enzymes or fluorescent molecules inside or within the lipid bilayer.

4. Specific ligand binding proteins included in the transport of small molecules. An example of this is the periplasmic binding proteins of bacteria which have been shown to bind many amino acids, glucose, galactose, ribose and other sugars, Pardee, A. Science, 162:632-637, (1968); G. L. Hazelbaur, and J. Adler, Nature New Bio. 230:101-104, (1971).

In the above-mentioned examples the ligand bound to the nucleic acid reacts with a naturally occurring protein. The specificity of this reaction resides in the ligand-binding site of the protein.

One further example of small molecule interaction with naturally occurring proteins involves the specific

binding of coenzyme or other prosthetic molecules to enzymes. Examples of such coenzymes include thiamin pyrophosphate, flavine mononucleotide, flavine adenine dinucleotide, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, coenzyme A, pyridoxyl phosphate, biotin, tetrahydrofolic acid, coenzyme B_{12}, lipoic and ascorbic acid. Many of these molecules form covalent linkages with their apoenzymes.

However, some, for example, coenzyme A, coenzyme B_{12} and tetrahydrofolic acid, associate in a non-covalent but specific manner with their cognate apoenzymes. A specific coenzyme-apoenzyme system for use in this system is flavine adenine dinucleotide (FAD) and flavine adenine dinucleotide reductase isolated from Escherichia coli. With this system the binding of FAD is sufficiently strong to permit detection.

The special nucleotides of this invention and polynucleotides including such nucleotides, either single-stranded or double-stranded polynucleotides, DNA and/or RNA, comprising the components, phosphoric acid moiety P, the sugar or monosaccharide moiety S, the base moiety B, a purine or a pyrimidine, and the signalling or self-detecting moiety, Sig, covalently attached to either the P, S or B moieties, as indicated hereinabove, have many uses and utilities. For example, the nucleotides of this invention and polynucleotides containing the nucleotides of this invention are useful as immune-stimulating agents, as adjuvants in vaccines, as agents for the stimulation or induction from competent cells, such as lymphocytes, for the production of lmphokines, cytokines or cytokinins, interferon or other cellular products.

It is well known that double-stranded poly A:U is a stimulator or inducer for the production of interferon, although weakly so. Similarly, poly I:C is also known as a stimulator or inducing agent for the production of interferon.

The advantage of polynucleotides, such as double-stranded polynucleotides incorporating one or more nucleotides in accordance with this invention is that, in effect, such polynucleotides would be more effective and more powerful inducing or stimulating agents for the production of interferon and related materials from cells. For example, nucleotides in accordance with this invention containing the above-described components P, S, B and Sig, are suitably prepared so that the nucleotides and polynucleotides prepared therefrom are more resistant to nucleases. Similarly, such nucleotides and polynucleotides containing the same and suitably prepared which are more capable of contacting, stimulating and penetrating cellular surfaces or membranes, such as the cellular surfaces or membranes of lymphocytes and other cells so as to stimulate the same for the production of a desired cellular product, such as interferon.

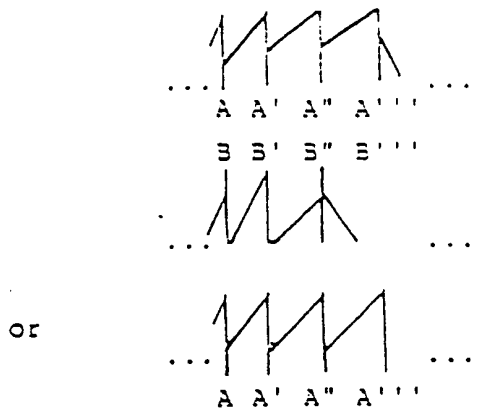
Particularly useful among those special nucleotides in accordance with this invention having the formula P-S-B-Sig and especially useful are those wherein the Sig component is at the 5 position of the pyrimidine or the 7 position of the purine or a deazapurine or the N^2 position of guanine or the N^6 position of adenine. Such nucleotides and polynucleotides incorporating the same, both single-stranded and double-stranded nucleotides, DNA and/or RNA are prepared in accordance with this invention to provide increased stability with respect to the double-stranded helix of DNA or RNA or DNA-RNA hybrid containing the same. Increased resistance to nucleases is also achievable as well as alterations or favorable changes in the hydrophobic properties or electrical or charge properties of the nucleotides and polynucleotides containing the same. Also, nucleotides and polynucleotides in accordance with this invention are prepared which, when administered to humans, have reduced pyrogenicity or exhibit less other whole body toxic responses. Additionally, the nucleotides and polynucleotides are prepared in accordance with this invention to provide a ligand, such as the component Sig, to which specific polypeptides can combine to create or bring about the formation of RNA complexes. It is seen therefore that the nucleotides of this invention include the P, S, B and Sig components wherein the Sig is covalently attached to either the P, S or B moieties open up or provide a whole array of chemical agents having special biological effects including therapeutic effects and cytotoxic effects.

The special nucleotides of this invention, including polynucleotides containing these nucleotides, in addition to being stimulating or inducing agents for the production of cellular materials or products, such as interferons, lymphokines and/or cytokines, are also useful for their chemotherapeutic effect and for the preparation of chemotherapeutic agents based thereon but also for their cytotoxic effects and the production of cytotoxic agents based thereon. The moiety Sig attached to the special nucleotides of this invention containing the other moieties or components P, S, B provides a site per se for the attachment thereto, the Sig component, of special agents of known chemotherapeutic or cytotoxic effect. Such nucleotides could be introduced or administered to the subject being treated, e.g. human body or animal, so as to be incorporated into the DNA and/or RNA components of the body or cell so as to either interfere with the synthesis of the body or cellular DNA and/or RNA or to attack tumors or to, in effect, kill or otherwise interfere with the growth of undesired cells.

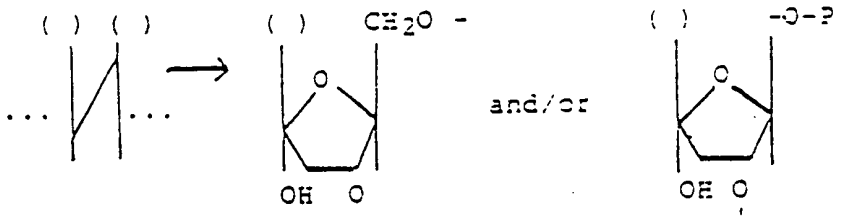
The administration of the nucleotides and/or polynucleotides containing the nucleotides to the body, human body or animal, can be effected by a number of suitable means. Particularly effective would be the

intravenous introduction to the body of preparations containing the nucleotides of this invention and a suitable physiologically acceptable carrier or the nucleotides could be administered subcutaneously, transdermally, or intramuscularly or by direct introduction into the site where the chemotherapeutic or cytotoxic effect of the nucleotides is sought or desired to be effective. Not only could desired chemotherapeutic or cytotoxic effects be achieved systemically or locally but also, as indicated hereinabove, the special P, S, B and Sig-containing nucleotides of this invention, including polynucleotides containing such nucleotides, are useful as immune-stimulating agents and adjuvants therefor. Accordingly, vaccines containing the special nucleotides and polynucleotides in accordance with this invention can be prepared having improved effectiveness and versatility.

Of special interest in the practices of this invention improved polynucleotides incorporating the special nucleotides of this invention are provided as inducers or stimulating agents for the production of interferon. Such polynucletoides would be single-stranded or double-stranded ribonucleotides, dsRNA, having the structures,



where A and B are complementary base pairs, such as a purine, a 7-deazapurine or pyrimidine modified by the addition of an organic moiety Sig in accordance with the disclosures of this invention on the 5 position of the pyrimidine ring or the 7 position of the purine ring or the N^2 of guanine, or the N^6 of adenine or the N^4 of cytosine as described herein. The modifications of the polynucleotides at these positions lead to relatively undisruptive or non-disruptive double-stranded nucleic acid molecules as measured by rates of association and melting points. In the special polynucleotides of this invention employed as inducers of interferon and other cellular or humoral factors or components, such as lymphokines or cytokines, the following groups would be attached thereto as indicated by the formulas,



In the utilization of the special polynucleotides of this invention, such as the special dsRNA of this invention in the induction process for the production of interferon it has been demonstrated that DEAE-dextran facilitates this operation. It appears that since DEAE-dextran complexes with dsRNA and protects it for nuclease degradation, thereby enhancing interferons induction. It has also been noted that poly rC : rI is taken into cells more efficiently when complexed with DEAE-dextran. Accordingly, in the practices of this invention the hydrophobic properties and the ionic or electron charge properties of the special dsRNA of this invention are important factors and capable of manipulation in the applicability of these materials to induce interferon production. It has been observed that such conditions or factors which promote the induction of interferon also lead to and promote the induction of other cellular or humoral components, such

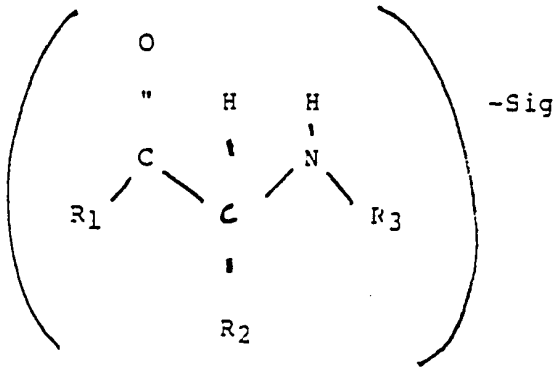
as lymphokines and cytokines. It is apparent, therefore, that the special nucleotides and polynucleotides containing the special nucleotides of this invention act as immune modulators and stimulators of the immune response other than simply being effective as inducers of interferon production. Superior agents for the above in accordance with the practices of this invention would include nucleotides wherein the Sig moiety incorporates biotin or streptavidin or avidin.

Poly rI:poly rC complexed poly L-lysine exhibits adjuvant properties and such properties are enhanced and improved in accordance with the practices of this invention when the poly rI and poly rC components are modified to include one or more of the special nucleotides in accordance with this invention.

The preparation of DNA probes in accordance with another aspect of this invention can be carried out in a manner which does not require the preparation or utilization of the special nucletoides described herein. For example, double-stranded DNA can be reacted with a carcinogen or alkylating agent. After the carcinogen has reacted with or alkylated the double-stranded DNA, the resulting modified DNA is melted to produce a DNA hybridizing probe containing the reaction product of the DNA and the carcinogen or alkylating agent. When thus-modified or reacted DNA is employed as a hybridizing probe, any resulting formed double helix or double-stranded DNA would be assayed or searched out by means of a double antibody technique. The primary antibody would be an anti-carcinogen and the secondary antibody would be horseradish-peroxidase conjugated anti-peroxidase antibody. The advantage of this rechnique is that it would be easy to label the double-stranded DNA. This special approach is indicated hereinabove in the examples accompanying the description of this invention and is generally applicable for the preparation of DNA probes from double-stranded or double helical DNA. However, this procedure is a disruptive procedure involving the modification of the double helical deoxyribonucleotide polymer or DNA.

In the description of the special nucleotides and modified DNA employed or developed in the practices of this invention, mention has been made of mono, oligo and polysaccharides. It is pointed out that derivatives of mono, oligo and polysaccharides are also useful in the preparation of the special nucleotides of this invention. For example, it is possible to modify individual sugar moieities employed in the make-up of the special nucleotides and employ the resulting modified sugar moieties to effect or carry out additional chemical reactions. Such modified mono, oligo and polysaccharide moieties, when employed as the Sig moieity in the preparation of the special nuleotides of this invention, provide an added versatility with respect to the detection of the nucleotides or other compounds containing such modified saccharides either as the sugar S or as the Sig moiety thereof.

In another aspect of this invention the Sig moiety instead of being attached to a nucleotide could also be attached to proteins. Not only could such proteins be attached to nucleotides or polynucleotides but also such proteins could be identified per se whether attached to a nucleotide or polynucleotide or unattached. In accordance with the practices of this aspect of the invention, a suitable such protein adduct would have the formula,



wherein R_1 is an OH or an amino acid or acids and R_2 is an amino acid side chain and R_3 is H or an amino acid or acids and Sig is attached to the R_1 and/or R_2 and/or R_3.

Claims

1. A nucleotide having the formula

Sig-P-S-B

wherein P is a phosphoric acid moiety, S is a sugar moiety and B is a pyrimidine or 7-deazapurine moiety, P being attached to the 3' and/or 5'position of the sugar moiety when said nucleotide is a deoxyribonucleotide and at the 2', 3' and/or 5' position when said nucleotide is a ribonucleotide, B being attached to the 1' position of S from the N1 position when B is a pyrimidine or the N9 position when B is a purine or 7-deazapurine, and Sig is covalenty attached to P directly or via the chemical linkage

said Sig, when attached to P does not interfere substantially with the characteristic ability of Sig to form a detectable signal and represents a moiety which is detectable when said nucleotide is incorporated into a double-stranded nucleic acid duplex, said Sig being a monosaccharide, polysaccharide or oligosaccharide or biotin or iminobiotin, an electron dense component, a magnetic component, an enzyme component, a radioactive component, a chemiluminescent component or an antigen, hapten or antibody component.

2. The nucleotide of claim 1 wherein Sig is a sugar residue and said sugar residue is complexed with or attached to a sugar or polysaccharide binding protein.

3. The nucleotide of claim 1 wherein Sig includes a coenzyme.

4. A polynucleotide comprising at least one nucleotide in accordance with claim 1.

5. A polynucleotide comprising at least one nucleotide of claim 1 wherein said polynucleotide is attached to a polypeptide.

6. The polynucleotide of claim 5, wherein said polypeptide has at least one biotin, iminobiotin, antibiotin, antiiminobiotin, avidin, streptavidin or enzyme attached thereto.

7. Use of a nucleotide or polynucleotide according to one of the preceding claims for the manufacture of a chemotherapeutic agent for inhibiting RNA and/or DNA synthesis or function in an organism.

8. Use of a nucleotide or polynucleotide according to one of the preceding claims for the manufacture of a stimulating or inducing agent for the stimulation or induction of cells for the production of lymphokines, cytokines and/or interferon.

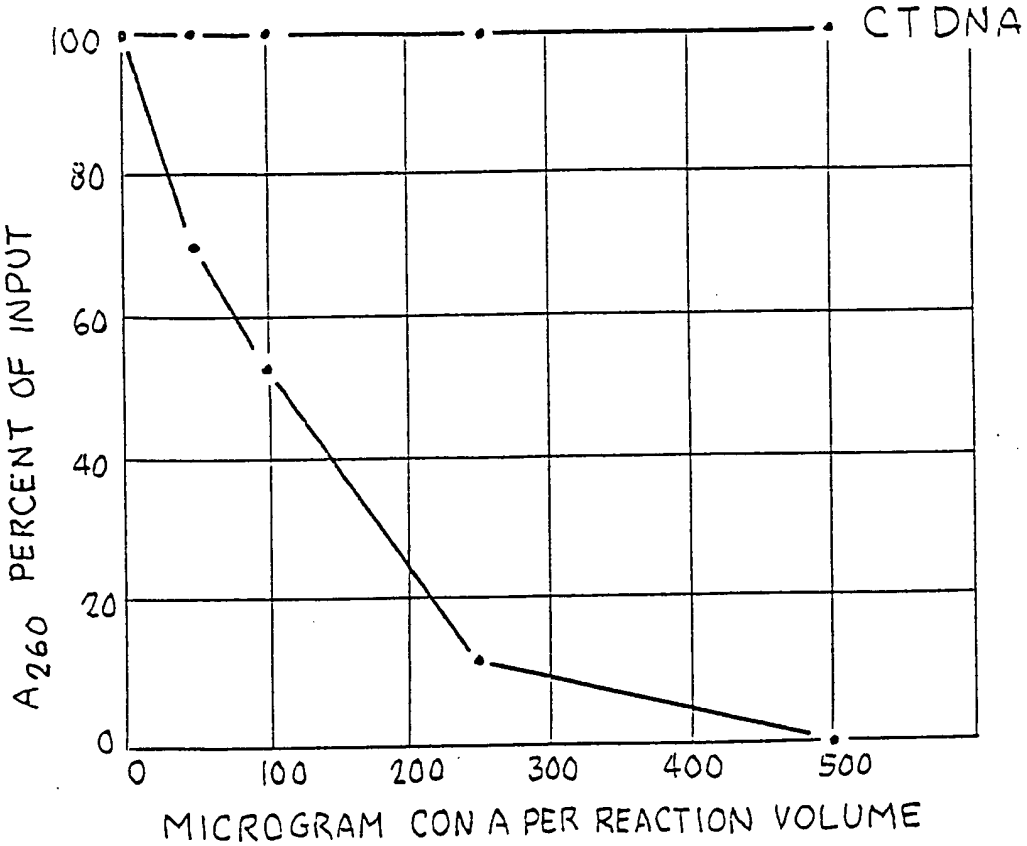
9. A method of detecting a first compound which includes a nucleotide in accordance with claim 1 as part of said first compound, which comprises contacting said first compound with a second compound capable of forming a complex therewith under suitable conditions so as to form said complex, said complex comprising said first compound and said second compound and detecting said complex.

10. A method of determining the presence of a deoxyribonucleic or ribonucleic acid molecule which comprises forming a double-stranded hybrid polynucleotide duplex which includes a single strand of deoxyribonucleic acid or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid molecule and a nucleotide in accordance with claim 1 and detecting said duplex.

11. A method of detecting the presence of a nucleic acid-containing etiological agent in a subject which comprises obtaining a suitable sample from said subject, detecting the presence in said sample of a deoxyribonucleic or ribonucleic acid naturally associated with said etiological agent by forming under suitable conditions a double-stranded polynucleotide duplex which includes a compound in accordance with claim 1 and a single strand of deoxyribonucleic or ribonucleic acid corresponding to or derived from said deoxyribonucleic or ribonucleic acid which is naturally associated with said etiological agent under suitable conditions and detecting the presence of said double-stranded polynucleotide duplex.

12. A method of testing a bacterium to determine its resistance to an antibiotic which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said bacterium which confers resistance of said bacterium to said antibiotic and which includes a nucleotide in accordance with claim 1 incorporated therein, contacting said polynucleotide under suitable conditions with a deoxyribonucleic acid obtained from said bacterium so as to form a double-stranded hybrid duplex and

Fig. 1.


CTDNA
100
80
60
40
20
0
A_{260} PERCENT OF INPUT
0
100
200
300
400
500
MICROGRAM CON A PER REACTION VOLUME

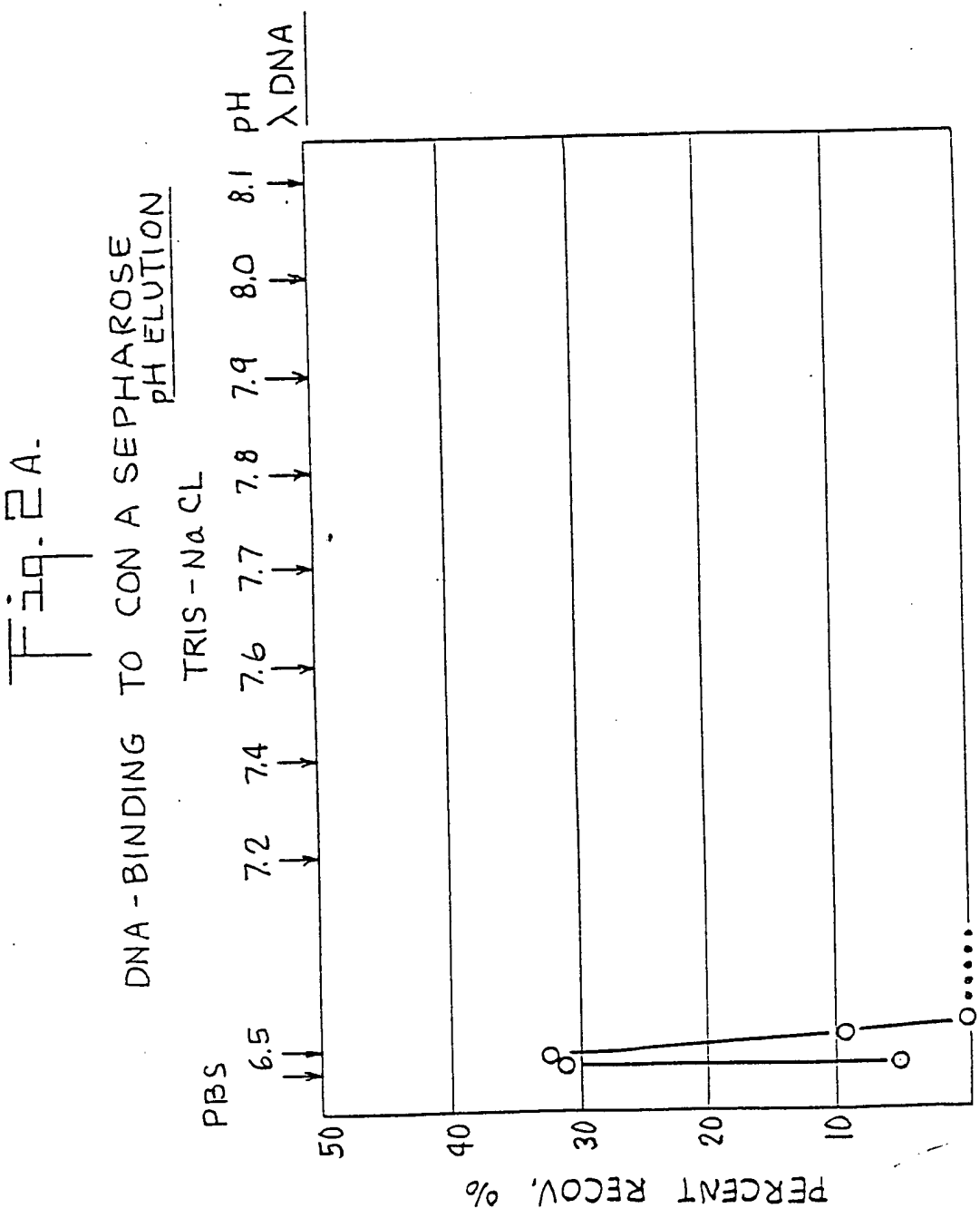
detecting the presence of said duplex, the detection of said duplex indicating resistance of said bacterium to said antibiotic and the absence of said duplex indicating the susceptibility of said bacterium to an antibiotic.

13. A method of diagnosing a genetic disorder in a subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence of said subject which is associated with said genetic disorder and includes a compound in accordance with claim 1 incorporated therein, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and detecting the presence of said hybrid duplex, the presence or absence of said hydrid duplex indicating the presence or absence of said genetic disorder.

14. A method of diagnosing thalassemia in a human subject which comprises preparing a polynucleotide complementary to the deoxyribonucleic acid gene sequence which is absent in β-minus-thalassemia subjects and includes a nucleotide in accordance with claim 1, contacting said polynucleotide under suitable conditions with deoxyribonucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and determining the presence of said duplex, the absence of said duplex indicating the presence of β-minus-thalassemia.

15. A method of chromosomal karyotyping which comprises preparing a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, said polynucleotides including one or more compounds in accordance with claim 1, contacting said polynucleotides with deoxyribonucleic acid of or obtained from chromosomes so as to form hybrid duplexes and detecting said duplexes, thereby determining the location of said duplexes on said chromosomes and the location of said genetic sequences on said chromosomes.

Fig. 2A.


DNA-BINDING TO CON A SEPHAROSE
pH ELUTION
TRIS-NaCL
PBS
6.5
7.2
7.4
7.6
7.7
7.8
7.9
8.0
8.1 pH
λ DNA
PERCENT RECOV, %
50
40
30
20
10

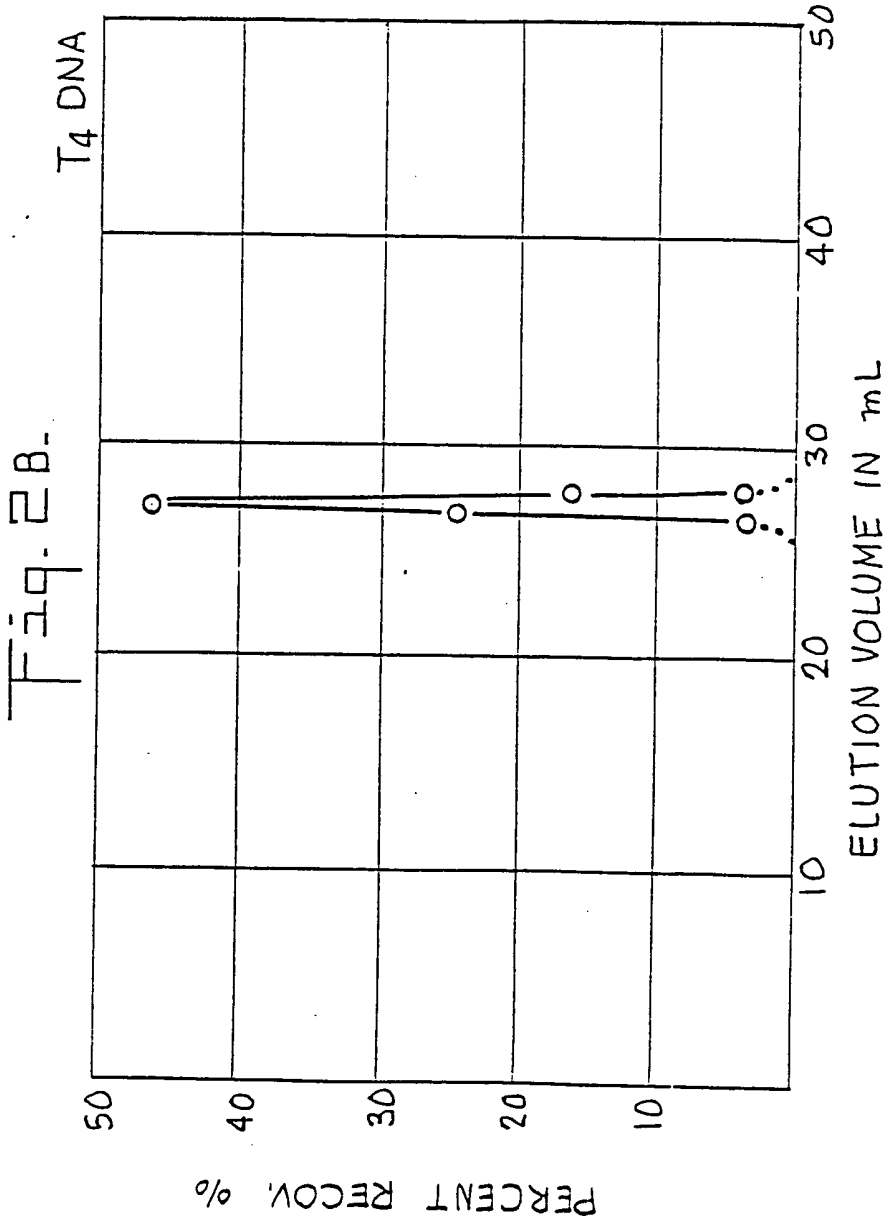

T4 DNA
PERCENT RECOV. %
50
40
30
20
10
ELUTION VOLUME IN mL
10
20
30
40
50

Fig. 2B.

Fig. 3A.

DNA BINDING TO CON A SEPHAROSE
EFFECT ON MANNOSE

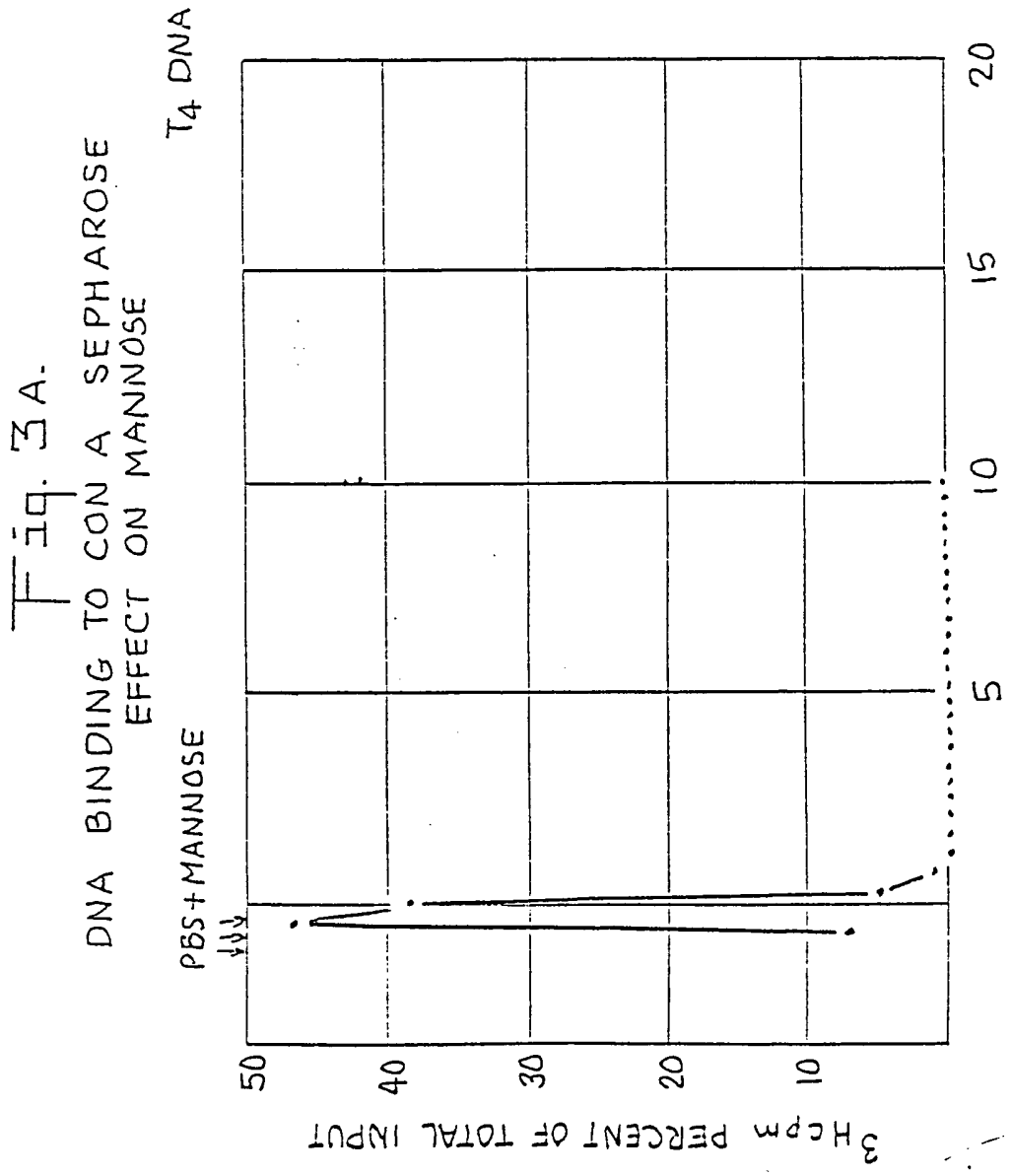


Fig. 3B.

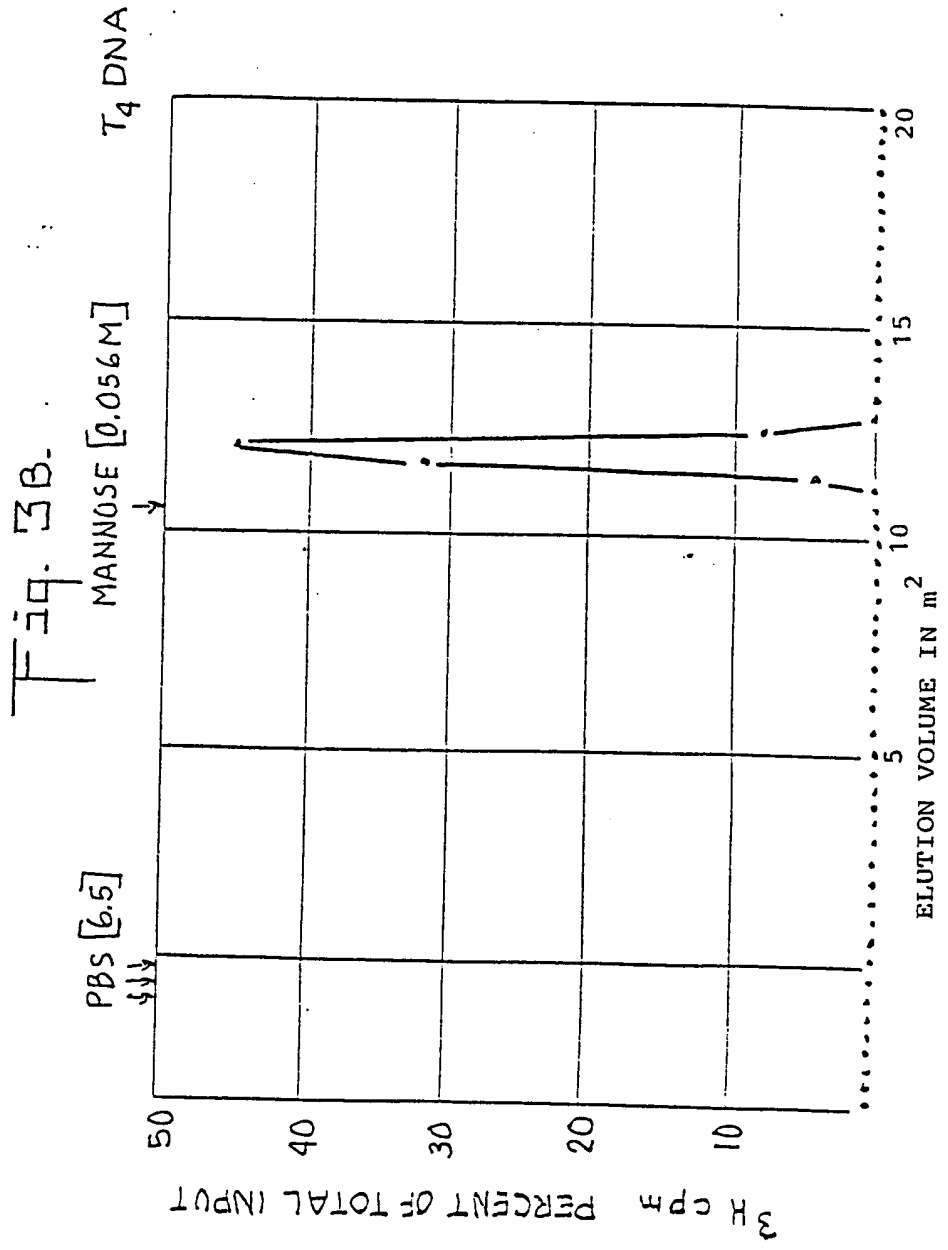

T4 DNA
MANNOSE [0.056M]
PBS [6.5]
3H cpm PERCENT OF TOTAL INPUT
50
40
30
20
10
5
10
15
20
ELUTION VOLUME IN m2

Fig. 4A.

CON-A SEPHAROSE BINDING OF GLUCOSYLATED DNAS
3/8 -9/82

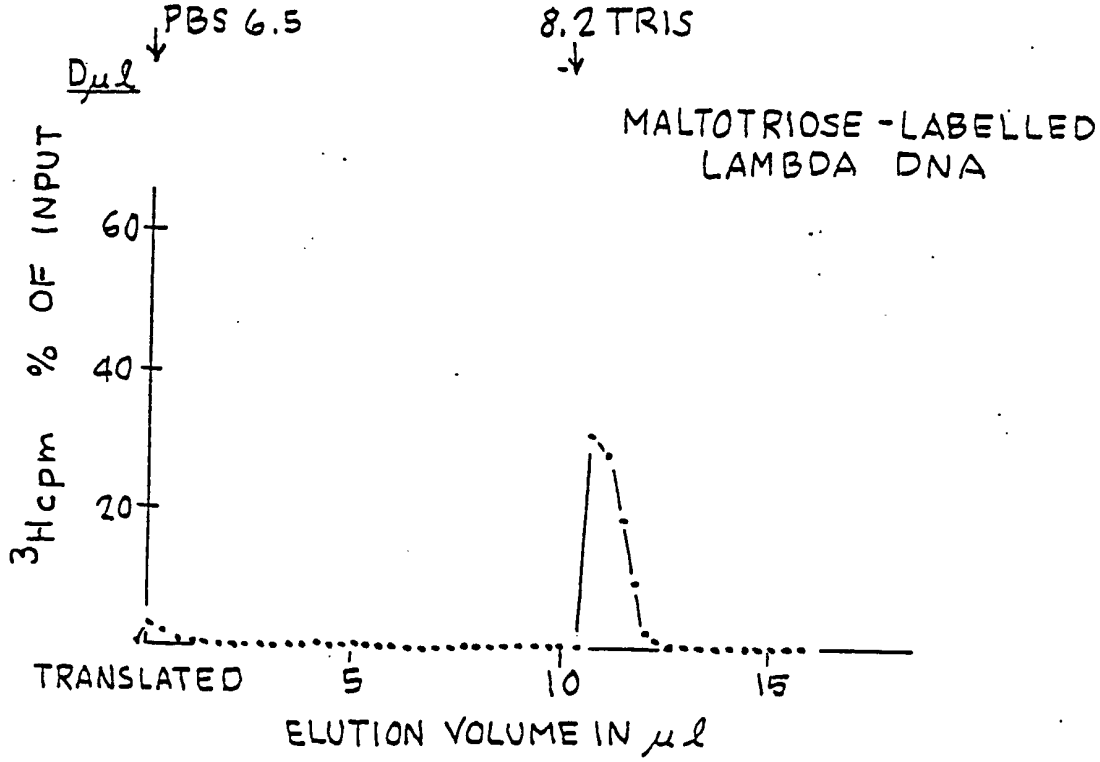
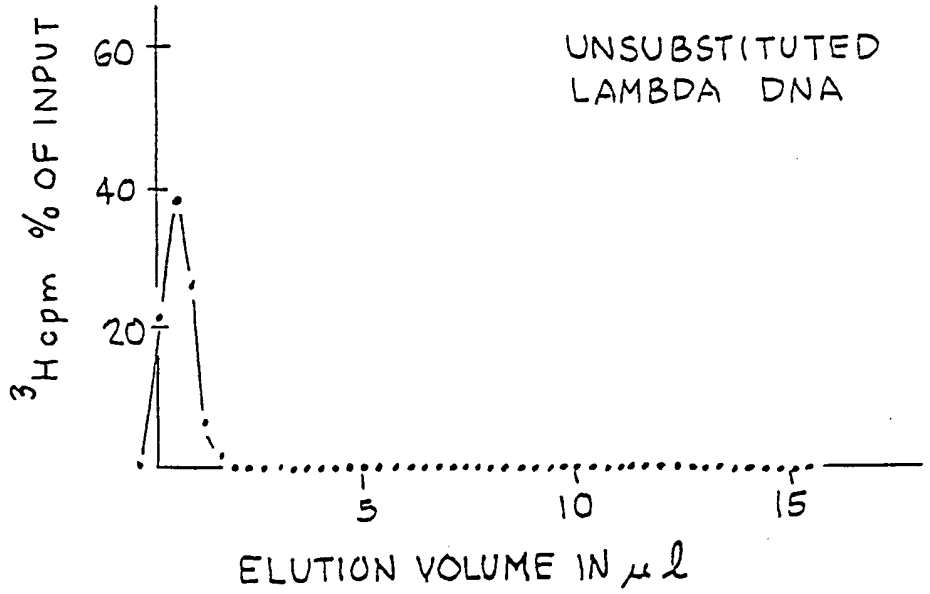


Fig. 4B.


UNSUBSTITUTED
LAMBDA DNA
^{3}H cpm % OF INPUT
60
40
20
5
10
15
ELUTION VOLUME IN μl

VOSSIUS & PARTNER

Mail Address: P. O. Box 860767 8000 München 86

Enzo Biochem, Inc.
Patent Department
Attn.: Mrs. Elaine P. Brenner
Corporate Patent Counsel
345 Hudson Street

NEW YORK, NY 10014
U.S.A.

PATENTANWÄLTE
EUROPEAN PATENT ATTORNEYS
Dr. VOLKER VOSSIUS, Dipl.-Chem.
Dr. PAUL TAUCHNER, Dipl.-Chem.
Dr. DIETER HEUNEMANN, Dipl.-Phys.
Dr. PETER A. RAUH, Dipl.-Chem.
Dr. GERHARD HERMANN, Dipl.-Phys.
JOSEF SCHMIDT, Dipl.-Ing.
Dr. HANS-RAINER JAENICHEN, Dipl.-Biol.
RECHTSANWÄLTIN
HELGA TREMMEL

SIEBERTSTRASSE 4
8000 MÜNCHEN 80
GERMANY (F.R.G.)
TELEPHONE: (089) 47 40 75
CABLE: BENZOLPATENT MÜNCHEN
TELEX: 529453 VOPAT D
TELEFAX: (089) 47 20 01

EP-Patent Application
No. 88 10 4962.1
ENZO BIOCHEM INC.
Your Ref.: Enz-5 (EPO) Div. V
Our Ref.: S 458 EP IV

August 13, 1990
DP/NH

Dear Mrs. Brenner,

Please find enclosed the European search report which has been drawn up for all claims.

Under separate cover, you will receive a copy of each of the references cited therein. Each category of the cited documents is explained at the bottom of the search report.

The main purpose of the search report is to give the applicant the opportunity to study the prior art in order to determine whether it is worthwhile pursuing the application further.

It is not obligatory to file any comments or revised documents in reply to the search report. The modification of the description and claims may be postponed until substantive examination proceedings.

If you wish us to comment in detail on the relevance of the documents cited in the European search report, please let us know.

Very truly yours,

VOSSIUS & PARTNER

Enc.
European search report

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Division de la recherche

P.B. 5[illegible]ntlaan, 2
2280[illegible] RIJSWIJK (ZH)
Pays-Bas / Netherlands / Niederlande

Telex 31651
(070) 340-20 40
BREVPATENT

Vossius & Partner
Siebertstrasse 4
P.O. Box 86 07 67
D-8000 München 86
REPUBLIQUE FEDERALE D'ALLEMAGNE

EINGEGANGEN
Vossius & Partner
8. AUG. 1990

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Datum/Date
- 6 AOUT 1990

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°.//Patent Nr./Patent No./Brevet n°.
S 458 EP Div. V	88104962.1- -

Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire
ENZO BIOCHEM, INC.

COMMUNICATION

The European Patent Office herewith transmits

- [X] the European search report
- [ ] the declaration under Rule 45 of the European Patent Convention
- [ ] the partial European search report under Rule 45 of the European Patent Convention
- [ ] the supplementary European search report concerning the international application number

relating to the above-identified European patent application; copies of the documents cited in the search report are enclosed.

The Search Division approved the following items, as submitted by the applicant:

[X] Abstract [X] Title [ ] Figure

- [ ] The abstract was modified by the Search Division and the definitive text is attached to the present communication.
- [ ] The following figure will be published with the abstract, since the Search Division considers that it better characterises the invention than the one indicated by the applicant.

  Figure:

- [X] Additional copy(ies) of the documents cited in the European search report.

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REFUND OF THE SEARCH FEE

If applicable under Art.10 of the Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent to you later.

F2P0109E.O1P0190.P2P0104

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 10 4962

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	US-A-4 310 662 (R. CREA) * Whole document * ---	1,4	C 07 H 21/00 C 07 H 19/073 C 07 H 19/10 C 07 H 19/14
X	BIOCHEMISTRY, vol. 16, no. 20, October 1977, pages 4478-4483, Washington, DC, US; J.K. MACKEY et al.: "Preparation and characterization of highly radioactive in vitro labeled adenovirus DNA and DNA restriction fragments" * Whole document * ---	1,4,10	
X	LIEBIGS ANNALEN DER CHEMIE, vol. 712, 1968, pages 195-200, Weinheim, DE; F. KATHAWALA et al.: "Darstellung von Desoxy-oligonucleotiden mit 2'.3'-[2.4-Dimethoxy-benzyliden]-uridin als Phosphat-Schutzgruppe" * Whole document * ---	1	
X	CARBOHYDRATE RESEARCH, vol. 102, May 1982, pages 139-146, Elsevier Science Publishing Co., Amsterdam, NL; M.A. SALAM et al.: "Synthesis of nucleoside 5'-(beta-D-glucopyranosyl monophosphates) by the sugar ortho ester route" * Whole document * ---	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 07 H 21/00
A	CELL, vol. 9, September 1976, pages 81-90, MIT, New York, US; L. ANGERER et al.: "An electron microscope study of the relative positions of the 4S and ribosomal RNA genes in HeLa cell mitochondral DNA" * Page 81, column 2, lines 26-42; page 87, column 2, lines 36-54; page 88, column 2, lines 45-56 * --- -/-	1,3,9, 10	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10-04-1990	WATCHORN P.W.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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EPO FORM 1503 03.82 (P0401)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 10 4962

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P,X	NUCLEOSIDES & NUCLEOTIDES, vol. 1, no. 2, 1982, pages 155-161, Marcel Dekker, Inc., New York, US; M.A. SALAM et al.: "Synthesis of acetylated alpha- and beta-L-fucosyl esters of nucleoside 5'-monophosphates by the orthoester route" * Whole document * ---	1	
P,X	EP-A-0 061 762 (BAKER INSTRUMENTS CORP.) * Whole document * ---	1	
P,X	EP-A-0 061 760 (BAKER INSTRUMENTS CORP.) * Whole document * ---	1	
P,X	EP-A-0 061 761 (BAKER INSTRUMENTS CORP.) * Whole document * -----	1	
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	10-04-1990	WATCHORN P.W.

CATEGORY OF CITED DOCUMENTS

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO.

EP 88 10 4962

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on 27/07/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		CA-A-	1167032	08-05-84
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		DE-A-	3278503	23-06-88
		JP-A,B	57177698	01-11-82

VOSSIUS & PARTNER

Patentanwälte

Vossius & Partner POB 86 07 67 81634 München Germany

Enzo Biochem, Inc.
Patent Department
Attn.: Ronald C. Fedus, Esq.
527 Madison Avenue
9th Floor

NEW YORK, NY 10022
U.S.A.

PATENTANWÄLTE
EUROPEAN PATENT ATTORNEYS
EUROPEAN TRADEMARK ATTORNEYS
Dr. VOLKER VOSSIUS, Dipl.-Chem.
(bis 1992; danach in anderer Kanzlei)
Dr. PAUL TAUCHNER, Dipl.-Chem.
Dr. DIETER HEUNEMANN, Dipl.-Phys.
Dr. PETER A. RAUH, Dipl.-Chem.
Dr. GERHARD HERMANN, Dipl.-Phys.
JOSEF SCHMIDT, Dipl.-Ing.
Dr. HANS-RAINER JAENICHEN, Dipl.-Biol.
Dr. ALEXA VON UEXKÜLL, M.Sc.
Dr. RUDOLF WEINBERGER, Dipl.-Chem.
Dr. WOLFGANG BUBLAK, Dipl.-Chem.
AXEL STELLBRINK, Dipl.-Ing.
Dr. JOACHIM WACHENFELD, (Biol.)
EUROPEAN PATENT ATTORNEYS
Dr. RENATE BARTH, Dipl.-Chem.
Dr. URSULA ENGLBRECHT, Dipl.-Chem.
RECHTSANWÄLTE
HELGA TREMMEL
BARBARA GUGGENMOS, Dipl.-Chem.

SIEBERTSTRASSE 4
81675 MÜNCHEN
GERMANY
TELEFON: +49-89-41 30 40
FAX: +49-89-41 30 41 11 (G3/G4)
+49-89-41 30 44 00 (G3)
(Marken - Trademarks)

NEU/NEW

EP-Patent Appln. No. 88 10 4962.1
ENZO BIOCHEM, INC.
Your Ref.: Enz-5 (EPO)(DIV. V)
Our Ref.: S 458 EP/V

December 7, 1998
Ba/ne

Dear Ronald,

Please find enclosed for your information copy of our today's letter to John J. Santalone, with enclosures.

Very truly yours,

Renate Barth

Dr. Renate Barth

Encl.

VOSSIUS & PARTNER

Patentanwälte

Copy

Vossius & Partner POB 86 07 67 81634 München Germany

Mr. John J. Santalone
Attorney at Law
488 Madison Avenue, 19th Floor

NEW YORK, NY 10022
U.S.A.

PATENTANWÄLTE
EUROPEAN PATENT ATTORNEYS
EUROPEAN TRADEMARK ATTORNEYS
Dr. VOLKER VOSSIUS, Dipl.-Chem.
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Dr. GERHARD HERMANN, Dipl.-Phys.
JOSEF SCHMIDT, Dipl.-Ing.
Dr. HANS-RAINER JAENICHEN, Dipl.-Biol.
Dr. ALEXA VON UEXKÜLL, M.Sc.
Dr. RUDOLF WEINBERGER, Dipl.-Chem.
Dr. WOLFGANG BUBLAK, Dipl.-Chem.
AXEL STELLBRINK, Dipl.-Ing.
Dr. JOACHIM WACHENFELD, (Biol.)
EUROPEAN PATENT ATTORNEYS
Dr. RENATE BARTH, Dipl.-Chem.
Dr. URSULA ENGLBRECHT, Dipl.-Chem.
RECHTSANWÄLTE
HELGA TREMMEL
BARBARA GUGGENMOS, Dipl.-Chem.

SIEBERTSTRASSE 4
81675 MÜNCHEN
GERMANY
TELEFON: +49-89-413040
FAX: +49-89-41304111 (G3/G4)
+49-89-41304400 (G3)
(Marken - Trademarks)

December 7, 1998
Ba/ne

EP-Patent Appln. No. 88 10 4962.1
ENZO BIOCHEM, INC.
Your Ref.: Enz-5 (EPO)(DIV. V)
Our Ref.: S 458 EP/V

Dear John,

Please find enclosed a further Official Communication issued by the European Patent Office in the above case. A reply is due by

February 16, 1999.

Please apologize the late transmittal of the Official Communication.

The Examiner holds the view that the revised claims submitted with our last reply meet the requirements of Art. 123(2) EPC and confirms their patentability with regard to novelty and inventive step. However, he has several objections with regard to clarity and also objects to claim

32 alleging that it is directed to a method of treatment. Furthermore, he requests the re-arrangement of the claims in groups relating to variants of a certain feature.

We enclose a draft of a new claims set which takes into account the Examiner's objections and suggestions and wherein we have also introduced multiple back references.

Please advise whether you can agree to our proposal or whether you suggest further amendments. The adaptation of the description to the new claims can be taken care of at our end, if you agree.

We are looking forward to receiving your instructions in due course, preferably not later than the beginning of February 1999.

Very truly yours,

Renate Barth

Dr. Renate Barth

Encl.
Official Communication
Draft of a new claims set

cc: Ronald C. Fedus, Esq.

EPA/EPO/OEB
D-80298 München
(089) 2399-0
TX 523 656 epmu d
FAX (089) 2399-4465

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Generaldirektion 2 | Directorate General 2 | Direction Générale 2

Telephone Numbers:
Primary Examiner (substantive examination) (089) 2399-8704
Formalities Officer / Assistant (Formalities and other matters) (089) 2399-8051

VOSSIUS & PARTNER
Postfach 86 07 67
81634 München
ALLEMAGNE

EINGEGANGEN
Vossius & Partner GbR
07. Okt. 1998
Frist bearb.:

Application No.	Ref.	Date
88 104 962.1-2105	S 458 EP Div. V	06. 10. 98

Applicant
ENZO BIOCHEM, INC.

Communication pursuant to Article 96(2) and Rule 51(2) EPC

The examination of the above-identified application has revealed that it does not meet the requirements of the European Patent Convention for the reasons enclosed herewith. If the deficiencies indicated are not rectified the application may be refused pursuant to Article 97(1) EPC.

You are invited to file your observations and insofar as the deficiencies are such as to be rectifiable, to correct the indicated deficiencies within a period

of 4 months

from the notification of this communication, this period being computed in accordance with Rules 78(3) and 83(2) and (4) EPC.

Amendments to the description, claims and drawings are to be filed where appropriate within the said period in **three copies** on separate sheets (Rule 36(1) EPC).

Failure to comply with this invitation in due time will result in the application being deemed to be withdrawn (Article 96(3) EPC).

EUROPÄISCHES PATENTAMT · EUROPEAN PATENT OFFICE · OFFICE EUROPÉEN DES BREVETS

KAAS V G
Primary Examiner
for the Examining Division

Enclosure(s): 2 page/s reasons (Form 2906)

Registered Letter

The examination is being carried out on the **following application documents**:

Text for the Contracting States:
AT BE CH LI DE FR GB IT LU NL SE

Description, pages:

1-120 as originally filed

Claims, No.:

1-39 as received on 04.02.1997 with letter of 04.02.1997

Drawings, sheets:

1/7-7/7 as originally filed

****

1) The new set of revised claims 1-39 appears to meet the requirements of Article 123(2) EPC. As already pointed out in the previous Official Communications, the said claims could form a basis for acceptance under Articles 52(1), 54 and 56 EPC. However, before a grant can be considered, the following points should be addressed.

2) The expression "and attached to P" in claim 1 appears to be superfluous in view of the formula given therein. The said expression should therefore be deleted (Article 84 EPC; conciseness).

3) Claims 35, 37 and 39 lack clarity (Article 84 EPC) due to the vague expression "which includes a compound in accordance with claim 1" . Given that the word "compound" is not recited in claim 1, the skilled reader would not be able to understand to what exactly said claims refer to. Clarification is therefore necessary.

4) All of the features recited in dependent Claim 5 are already recited in Claim 1. For the sake of conciseness, Claim 5 should therefore be deleted (Article 84 EPC).

5) The claims should be rearranged in order to enable the association between related claims to be more readily determined (Rule 29(4) EPC; see also the Guidelines, C-III, 3.6). In the present case, it would appear appropriate to group together e.g. the claims relative to the kind of linkage, i.e. renumber present claim 10 as claim 3, present claim 3 as claim 5, etc. Similarly, other claims relative to variants of a certain feature should also be grouped together.

6) Claim 32 encompasses a method of therapeutical treatment which is explicitely excluded from patentability by the provisions of Article 52(4) EPC. The said claim should therefore be either deleted or amended as indicated in the Guidelines C-IV, 4.2, last paragraph.

7) The Applicant is requested to bring the description into conformity with the claims; care should be taken during revision, especially of the introductory portion including any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed, Article 123(2) EPC. An additional copy showing the handwritten amendments would be appreciated.

VOSSIUS & PARTNER
PATENTANWÄLTE
SIEBERTSTR. 4
81675 MÜNCHEN

04. Feb. 1997

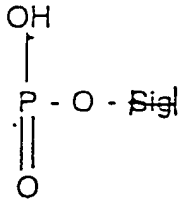
1. An oligo- or polynucleotide ~~nucleotide~~ comprising at least one nucleotide having the formula

Sig - P - S - B

wherein P is a phosphate moiety, S is a sugar moiety and B is a pyrimidine, purine or 7-deazapurine moiety, P being attached to the 3' or the 5' position of the sugar moiety when said nucleotide is a deoxyribonucleotide and at the 2', 3' or 5' position when said nucleotide is a ribonucleotide, B being attached to the 1' position of S from the N^1 position when B is a pyrimidine or the N^9 position when B is a purine or 7-deazapurine, and ~~Sig is covalently attached to P directly or via a chemical linkage, said~~ Sig being a detectable moiety ~~when~~ and attached to P.

2. The oligo- or polynucleotide of claim 1, wherein Sig is covalently attached to P directly or via a chemical linkage.

4. ~~2~~ The oligo- or polynucleotide of claim 2 or 3, wherein said ~~covalent~~ chemical linkage comprises



5. ~~3~~ The oligo- or polynucleotide of any one of claims 2 to 4, wherein said chemical linkage does not interfere substantially with the characteristic ability of Sig to form a detectable signal.

~~5. The oligo- or polynucleotide of claim 1, wherein the attachment~~ of Sig to P does not interfere substantially with ~~the characteristic~~ ability of Sig to form a detectable signal ~~and Sig~~ represents a moiety which is detectable when ~~said~~ oligo- or polynucleotide is incorporated, hybridized ~~or complexed in a double-stranded nucleic acid duplex~~.

25. The oligo- or polynucleotide of claim 24, wherein said nucleotide comprises a deoxyribonucleotide.

26. The oligo- or polynucleotide of claim 24, wherein said nucleotide comprises a ribonucleotide.

7. The oligo- or polynucleotide of any one of claims 1 to 6, wherein Sig is a moiety containing at least three carbon atoms.

8. The oligo- or polynucleotide of any one of claims 1 to 7, wherein Sig includes a glycosidic linkage moiety. .

~~9. The oligo- or polynucleotide of claim 4, wherein Sig is attached to P via a chemical linkage~~.

3. ~~10~~. The oligo- or polynucleotide of claim ~~8~~, wherein said chemical linkage comprises or includes an olefinic bond at the ∂-position relative to P, or any of the moieties:

$$-CH=CH_2-NH-$$

$$-CH=CH-CH_2-NH-$$

$$-CH=CH-CH_2-O-CH_2-\underset{\underset{OH}{|}}{CH}-CH_2-NH-$$

$$-S-,\ -\overset{\overset{O}{\|}}{C}-O \text{ and } -O- .$$

6. ~~11~~. The oligo- or polynucleotide of any one of claims 1 to 5 wherein Sig is terminally attached to the oligo- or polynucleotide.

9. ~~12~~. The oligo- or polynucleotide of any one of claims 1 to 8 wherein Sig comprises a component selected from the group consisting of biotin, iminobiotin, an electron dense component, a magnetic component, an enzyme, a hormone component, a radioactive component, a metal-containing component, a fluorescent component, a chemiluminescent component, an antigen, a hapten and an antibody component.

10. ~~13~~. The nucleotide of claim 9 ~~12~~, wherein said electron dense component comprises ferritin.

11. 9
~~14.~~ The oligo- or polynucleotide of claim ~~12~~, wherein Sig is <—> complexed with a binding protein therefor, and said binding protein is conjugated to ferritin.

12. 9
~~15.~~ The oligo or polynucleotide of claim ~~12~~, wherein Sig comprises a magnetic component.

13. 12
~~16.~~ The oligo- or polynucleotide of claim ~~12~~, wherein said magnetic component comprises a magnetic oxide.

14. 13
~~17.~~ The oligo- or polynucleotide of claim ~~16~~, wherein said magnetic oxide comprises ferric oxide.

15. 9
~~18.~~ The oligo- or polynucleotide of claim ~~12~~, wherein Sig comprises an enzyme.

16. 15
~~18.~~ The oligo- or polynucleotide of claim ~~18~~, wherein said enzyme is selected from the group consisting of alkaline phosphatase, acid phosphatase, ß-galactosidase, ribonuclease, glucose oxidase and peroxidase.

17. 9
~~20.~~ The oligo- or polynucleotide of claim ~~12~~, wherein Sig comprises a radioactive component.

<a sugar residue and said sugar residue is>

18. ~~21~~ 17. The oligo- or polynucleotide of claim ~~20~~, wherein said radioactive component is radioactive cobalt.

19. ~~22~~ 9. The oligo- or polynucleotide of claim ~~12~~, wherein Sig comprises a metal-containing component.

20. ~~23~~ 19. The oligo- or polynucleotide of claim ~~22~~, wherein said metal-containing component is catalytic.

21. ~~24~~ 9. The oligo- or polynucleotide of claim ~~12~~, wherein Sig comprises a fluorescent component.

22. ~~25~~ 21. The oligo- or polynucleotide of claim ~~24~~, wherein said fluorescent component comprises a member selected from the group consisting of fluorescein, rhodamine and dansyl.

23. ~~26~~ 9. The oligo- or polynucleotide of claim ~~12~~, wherein Sig comprises a chemiluminescent component.

24. ~~27~~ 9. The oligo- or polynucleotide of claim ~~12~~, wherein Sig comprises a hapten component capable of complexing with an antibody specific thereto.

27. ~~28~~ any one of claims 1 to 26 in single-stranded, The oligo- or polynucleotide of double-stranded or partially double-stranded form.

28.

~~29~~. Use of the oligo- or polynucleotide according to one of the preceding claims ~~as~~ for the preparation of a therapeutic agent for inhibiting RNA and/or DNA synthesis or function in an organism.

29.

~~30~~. Use of the oligo or polynucleotide according to one of the preceding claims as a stimulating or inducing agent for the stimulation or induction of cells for the production of lymphokines, cytokines and/or interferon.

<for the preparation of a pharmaceutical composition for use>

30.

~~31~~. Use of the oligo or polynucleotide according to one of the preceding claims <-> as an anti-tumor or cytotoxic agent.

31.

~~32~~. A method of detecting a first compound which includes an oligo- or polynucleotide in accordance with any one of claims 1 to 27 as part of said first compound, which comprises contacting said first compound with a second compound capable of forming a complex therewith under suitable conditions so as to form said complex, said complex comprising said first compound and said second compound and detecting said complex.

32.

~~33~~. A method of determining the presence of a nucleic acid molecule which comprises forming a double-stranded polynucleotide duplex with the nucleic acid molecule and includes the oligo- or polynucleotide of any one of claims 1 to 27 ~~and~~ detecting said duplex.

33.

~~34~~. A method of detecting the presence of a nucleic acid-containing etiological agent in a subject which comprises obtaining a suitable sample from said subject, detecting the presence in said sample of a nucleic acid naturally associated with said etiological agent by forming under suitable conditions a double-stranded polynucleotide duplex which includes ~~a compound~~ [-] in accordance with any one of claims 1 to 27 ~~and~~ a single-stranded nucleic acid corresponding to or derived from said nucleic acid

[an oligo- or polynucleotide]

which is naturally associated with said etiological agent, and detecting the presence of said double-stranded polynucleotide duplex.

34.
~~35.~~ A method of testing a bacterium to determine its resistance to an antibiotic which comprises preparing a polynucleotide complementary to the nucleic acid gene sequence of said bacterium which confers resistance of said bacterium to said antibiotic and which includes an oligo- or polynucleotide in accordance with ~~claims~~ anyone of 1 to 27 incorporated therein, contacting said polynucleotide with a nucleic acid obtained from said bacterium so as to form a double-stranded hybrid duplex and detecting the presence of said duplex, the detection of said duplex indicating resistance of said bacterium to said antibiotic and the absence of said duplex indicating the susceptibility of said bacterium to said antibiotic.

35.
~~36.~~ A method of diagnosing a genetic disorder in a subject which comprises preparing a polynucleotide complementary to the nucleic acid gene sequence of said subject which is associated with said genetic disorder and includes ~~a compound~~ [-] in accordance with ~~claims~~ anyone of 1 to 27 incorporated therein, contacting said polynucleotide under suitable conditions with nucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and detecting the presence of said hybrid duplex, the presence or absence of said hybrid duplex indicating the presence or absence of said genetic disorder.

36.
~~37.~~ A method of diagnosing thalassemia in a human subject which comprises preparing a polynucleotide complementary to the nucleic acid gene sequence which is absent in ß-minus-thalassemia subjects and includes an oligo- or polynucleotide in accordance with ~~claims~~ anyone of 1 to 27, contacting said polynucleotide under suitable conditions with nucleic acid obtained from said subject so as to form a double-stranded hybrid duplex and determining the presence of said duplex, the absence of said duplex indicating the presence of ß-minus-thalassemia.

[an oligo- or polynucleotide]

37.

~~38.~~ A method of chromosomal karyotyping which comprises preparing a series of modified polynucleotides corresponding to a series of defined genetic sequences located on chromosomes, said polynucleotides including one or more ~~compounds~~ in accordance with any one of claims 1 to 27, contacting said polynucleotides with nucleic acid of or obtained from chromosomes so as to form hybrid duplexes and detecting said duplexes, thereby determining the location of said duplexes on said chromosomes and the location of said genetic sequence on said chromosomes.

* * * * * * *

[oligo- or polynucleotides]

Volume 5 Number 2 February 1978 Nucleic Acids Research

Gene mapping and gene enrichment by the avidin-biotin interaction: use of cytochrome-c as a polyamine bridge

Ann Sodja and Norman Davidson

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA

Received 31 October 1977

ABSTRACT

A modification of previously described methods of electron microscopic gene mapping and of gene enrichment based on the avidin-biotin interaction is presented. The modification consists of coupling cytochrome-c instead of pentane diamine to the oxidized 2', 3' terminus of an RNA by Schiff base formation and BH_4^- reduction. The RNA-cytochrome-c conjugate is purified by a simple chromatographic procedure; several biotins are attached to the cytochrome moiety by acylation. The extended arm between biotin and RNA gives efficient electron microscopic gene mapping of DNA:RNA-biotin hybrids with avidin-ferritin and avidin-polymethacylate sphere labels and efficient gene enrichment by buoyant banding of DNA:RNA-biotin:avidin-spheres in CsCl. A 1400 fold enrichment (thus, 25% pure) and a 90% yield of long Drosophila DNA strands with 5S RNA genes is achieved.

INTRODUCTION

The preceding paper (1) describes a method of mapping, with a ferritin label, a short RNA:DNA hybrid region along a single stranded segment of DNA. The essential features of the method are: a) covalent attachment of biotin to the periodate oxidized 3' terminus of the RNA by a diamine bridge, using a simple diamine such as $NH_2(CH_2)_5NH_2$; b) covalent attachment of avidin to the electron opaque label ferritin; c) hybridization of the covalent tRNA-biotin conjugate to a single stranded segment of DNA that contains the coding sequence (gene) for the tRNA; d) electron microscopic mapping of the position of the hybridized tRNA-biotin along the single strand segment of DNA after binding of ferritin-avidin to the biotin. This method gives a moderately satisfactory overall efficiency of gene labeling, and has been used to map the tRNA genes of HeLa mitochondrial DNA (2). We describe here an improved method in which we use a defined polyamine instead of a diamine as the bridge between the 3' end of the RNA and the carboxylic acid biotin. The polyamine used is the protein, cytochrome-c. This bridge is believed to have several advantages: a) it is probably more extended than the pentane diamine; b) several biotins can be attached to one RNA molecule; c) the

purification step for tRNA-biotin with the pentane diamine bridge involves elution from avidin sepharose at pH 2.5 in 6M guanidine hydrochloride. These fairly drastic conditions are replaced for purification of tRNA-cytrochrome-c-biotin (tRNA-cc-biotin) by hydroxyapatite chromatography at neutral pH, thus diminishing the probability of chemical degradation of the labeled RNA. As a result, presumably, of items a, b, and/or c, the overall efficiency of gene labeling with ferritin in the electron microscope by the new method is an improvement over that achieved previously. d) with the new extended bridge but not with the diamine bridge, avidin attached to polymethylmethacrylate spheres (3) will label DNA:tRNA-biotin hybrids.

Furthermore, the binding of avidin-spheres by DNA:RNA-biotin molecules forms the basis for a gene enrichment procedure. The polymer spheres used have a density and molecular weight of about 1.25 g/ml and 7.6×10^7 daltons, respectively. The spheres can be used as relatively massive floats to separate DNA:RNA-biotin-avidin-sphere molecules from unhybridized DNA strands by buoyant banding in CsCl, thus accomplishing gene enrichment. Manning, Pellegrini and collaborators have demonstrated this application of the avidin-biotin labeling approach for long RNA molecules, by enriching for the rDNA genes of Drosophila and for the histone genes of the sea urchin (4,5). In these cases, it is appropriate and convenient to attach cytochrome-c-biotin nonspecifically to the RNA by CH_2O crosslinks. In the present communication, we show that 3' terminal labeling of 5S RNA by cytochrome-c can be used for highly efficient gene enrichment of the Drosophila 5S RNA genes.

The basic reaction scheme of the present procedure is:

1) Oxidation of free 2', 3' OH ends of RNA to the dialdehyde with periodate.

2) Schiff base formation of the terminal dialdehyde with the polyamine, cytochrome-c, at relatively low ionic strength, and stabilization of the compound against dissociation and/or β elimination by BH_4^- reduction.

3) Purification of RNA-cytochrome-c from free RNA and free cytochrome-c by sequential chromatography on carboxymethyl cellulose (CMC) and on hydroxyapatite (HAP).

4) Covalent attachment of several biotin molecules to lysine NH_2 groups of the cytochrome-c by acylation with the N-hydroxy succinimide (NHS) ester of the carboxylic acid biotin.

5) Hybridiaztion of the RNA-cc-biotin to DNA.

6) Labeling with avidin-ferritin or avidin-spheres.

7) Gene mapping by electron microscopy or gene enrichment by banding in CsCl.

MATERIALS AND METHODS

Nucleic Acids. E. coli tRNA's, ϕ80 and ϕ80 psu_3^- DNA's, were obtained as described (1). Drosophila (Dm) 4S and 5S RNA and the Drosophila plasmids, pCIT19 and pCIT12, were prepared as previously described (6,7). Unlabeled as well as ^{3}H-labeled Dm DNA's were isolated from Schneider's line 2 tissue culture cells. Cells were labeled by the addition of 1.5 m Ci of ^{3}H-thymidine (Amersham Radiochemical Center, 41 Ci/mmol) to 50 ml of cells grown in suspension with gentle swirling. Cell density at the first addition of label was 1-2 x 10^6 ml; label was added in four equal fractions at 6 hr intervals, and cells grown for another generation (approximately 24 hrs) after the last addition. Cells were harvested by centrifugation at 2400 rpm at 0°C for 5 minutes, lysed by homogenization (10-15 strokes) in 0.5 M Tris base 0.025 M KCl, 5 mM $Mg(Ac)_2$, 0.35 M sucrose, pH 7.6. DNA was prepared from this lysate by the procedure of Manning et al. (8). The specific activities of two separate preparations were 5.4 and 0.84 x 10^5 cpm/µg.

Cytochrome-c. Commercial cytochrome-c (horse heart, type VI, Sigma) is contaminated with RNAase, which has approximately the same molecular weight and charge. RNAase was inactivated by treatment with iodoacetate by a modification of published procedures (9,10). Cytochrome-c (60 mg) was dissolved in .1 ml of 0.2 M NaAc buffer (pH 5.5). An equal weight of iodoacetate was added, the pH readjusted to 5.5 with concentrated NaOH, and the solution diluted to a final volume of 2 ml. The solution was incubated for 1 hr at 55°C and then dialyzed extensively at 0°C against 0.01 M sodium phosphate buffer (NaP), pH 6.8, and lastly against 0.1 M $NaHCO_3/Na_2CO_3$ buffer, pH 9.2.

Preparation and Purification of RNA-Cytochrome-c. tRNA or 5S RNA were heated at 80° for 1-8 min in 1 mM NaAc buffer pH 6.8, cooled, adjusted to 0.1 M NaAc buffer (pH 4.8) and treated with periodate as previously described (1). The amount of RNA used was 0.5 - 1 mg in 0.5 - 1 ml of reaction mixture.

Oxidized RNA was dialyzed against 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.2) at 0°C. A 10-15 fold molar excess of cytochrome-c was added and the solution

ABBREVIATIONS

RNA-cc-biotin, 4S or 5S RNA-cytochrome-c-biotin; HAP, hydroxyapatite; CMC, carboxymethylcellulose; NHS-biotin, N-hydroxysuccinimide ester of biotin; NaP, sodium phosphate buffer (50:50 mono and dibasic sodium phosphate); Dm DNA or RNA, Drosophila melanogaster nucleic acids; EM, electron microscopy.

incubated for 1 hr at room temperature. A total of 2 mg $NaBH_4$/1 mg RNA was added in 4 portions over a period of 80 min. The solution was allowed to stand at room temperature for an additional 30 min and the $NaBH_4$ decomposed by addition of 0.1 - 0.2 ml of 4 M NaAc buffer (pH 5.0). The contents were dialyzed at 0°C against 0.01 M NaP buffer, pH 6.8. All of the steps up to and including reduction with $NaBH_4$ were performed in the dark.

For the spectrophotometric determination of concentration, we use molar extinction coefficients of 9.64×10^4 at 410 mμ, 5.4×10^5 and 8.3×10^5 at 260 mμ for cytchrome-c, 4S RNA, and 5S RNA, respectively.

Free cytochrome-c was removed from the reaction mixture by passage over a 3 x 1 cm column of carboxymethyl cellulose (CMC) that had been reequilibrated with 0.01 M NaP buffer. The CMC had been washed with acid and base according to the directions provided by the supplier. The sample was loaded and washed in 0.01 M NaP buffer. Free tRNA and the tRNA-cytochrome-c conjugate come through in the first wash.

Hydroxypatite (HAP, Bio-gel HTP from Bio-Rad) was hydrated by boiling for 10-20 min in 0.01 M NaP, pH 6.8, washed with 0.5 M NaP buffer, and reequilibrated in 0.01 M NaP. For each washing the suspension was swirled gently and allowed to settle for 10-15 minutes before decanting the finer particles. The HAP was packed into a 2 x 0.5 cm column in 0.01 M NaP and the mixture of tRNA and tRNA-cytochrome from the CMC column applied. The column was successively washed with 10 ml volumes of 0.1 M, 0.15 M, 0.3 M and 0.5 M NaP buffer, pH 6.8. Fractions (0.5 - 1 ml) were collected, and assayed by spectrophotometry.

Addition of Biotin. The N-hydroxysuccinimidyl (NHS) ester of ^{14}C-biotin was prepared as described (1). The 1:1 conjugate, tRNA-cytochrome-c, from the HAP column was treated with an approximately 100 fold excess of NHS-^{14}C-biotin under conditions previously described (3). Free biotin was removed by dialysis against 0.01 M NaP and the tRNA-cc-biotin stored at -20°C.

EM Labels. Ferritin-avidin was a gift from L. Angerer (1). Polymethylmethacrylate spheres (a gift from N.D. Hershey) were conjugated to avidin as previously described (3). One of the sphere-avidin preparations was a gift of M. Pellegrini.

Heteroduplex formation and electron microscopy. Heteroduplex formation between Dm plasmids containing 4S or 5S genes with DNA of the vector, Colicin E1, has been described (6,7).

φ80h/φ80 psu_3 heteroduplexes were formed as follows: a solution containing equal amounts of φ80h and φ80 psu_3 bacteriophage was treated with 20 μl

of 0.2 M EDTA (pH 8.0) for 30 min on ice. Complete lysis of the virions and denaturation of DNA was accomplished by addition of 20 µℓ of 1 N NaOH for 10-15 min at room temperature. The mixture was neutralized with 30 µℓ of 2.5 M Tris HCl. tRNA-cc-biotin was added and the volume was made to 200 µℓ with 3X recrystallized formamide (99%, Matheson, Coleman and Bell). The final DNA and tRNA concentrations were 3 µg/ml and 10-30 µg/ml. Hybridization was performed by dialysis of this mixture against 40% 3X recrystallized formamide, 0.1 M Tris, 0.3 M NaCl, 1 mM EDTA, pH 8.0 at 40°C for 40-50 min. Subsequent manipulations were as described (2). The concentration of spheres-avidin in labeling experiments was approximately 100 µg/ml. No removal of excess spheres-avidin was attempted, as an effective procedure to do so is not available.

All electron microscopy and measurements of molecular lengths were done as previously described (2, 6, 7). Single and/or double stranded ϕX 174 DNA (5370 nucleotides or nucleotide pairs, (11)), was used as a length standard.

Preparation of Dm ^{125}I 5S RNA. ^{125}I-5S RNA was prepared essentially according to Orosz and Wetmur (12). The reaction mixture contained, in the order of addition, the following: 10 µℓ H_2O (double distilled), 3-5 µℓ Dm 5S rRNA (1.9 mg/ml in 0.01 M NaAc, pH 4.8), 3 µℓ of 1 M NaAc (pH 5.0), 10 µℓ ^{125}I (Amersham, carrier free, 100 µCi/ml), and 3 µℓ of freshly prepared $TlCl_3$ (ICN-K & K Laboratories, 18 mg/10 ml double distilled H_2O). The mixture was incubated at 60° for 20 min in a sealed siliconized 50 µℓ pipette. The contents were transferred to 1 ml of 0.1 M NaAc buffer (pH 5.0) containing about 50-60 µg of Dm 18S and 28S rRNA, and dialyzed against 0.5 M NaCl, 0.015 M NaH_2PO_4, 2×10^{-4} M EDTA, pH 6.0 at 0° (2 x 500 ml) and at 60°C (2 x 500 ml) and at 0°C again until no counts were detected in the dialysate. The ^{125}I-5S RNA preparation was then treated with 50 µg/ml proteinase K (EM Laboratories, Inc.), phenol extracted, and further purified as described (4) or on Cs_2SO_4 gradients. Specific activities obtained in the different preparations ranged from $0.2 - 1 \times 10^8$ cpm/µg.

Solution Hybridization of ^{125}I-5S RNA to DNA fractions. The contents of 5S genes in the several fractions of Dm DNA for the gene enrichment experiments were carried out by saturation hybridization using excess ^{125}I-5S RNA in solution. DNA solutions were denatured in 0.2 M NaOH, neutralized, and adjusted to the 80% formamide hybridization solution described in the next section. All of the samples contained 10^{-4} M KI in order to reduce background. Typical concentrations of DNA assayed in the respective fractions were 0.075 - 0.5 µg/ml, 13-38 µg/ml, and 26-400 µg/ml, in the enriched, un-

fractionated, and depleted fractions, respectively. Reactions were carried out to a rot of 0.06 - 0.6 mol sec/liter. Samples were diluted 10 fold with 2 x SSC and treated at 37° for 1 hour with RNAase (100 µg/ml RNAase A, 4 units/ml T1 RNAase).

Gene Enrichment Procedure. RNA:DNA hybridization for the gene enrichment experiments was carried out in a high formamide solvent (13) which permits RNA:DNA hybridization but little or no DNA:DNA reassociation. Formamide was 3X recrystallized. DNA in 80% formamide, 2XSSC, was denatured by heating to 80°C for 10 min. A typical hybridization mixture contained 100 µg/ml Dm ^{3}H-DNA, 10 µg/ml Dm 5S RNA-cc-biotin, 150 - 1000 µg/ml Dm 18 + 28S rRNA, all in 2XSSC, 80% formamide at 45°C for 30 min (rot ≈ 6 x 10^{-2} mol sec l^{-1} for the 5S RNA). The sample was dialyzed at 0°C against 0.1 M NaCl, 1 mM Tris, 1 mM EDTA, pH 8.5, and passed over a Sepharose 2B column (19 x 1 cm) to remove excess 5S RNA-cc-biotin. Elution volume has previously been calibrated with Dm ^{3}H-DNA and ^{125}I 5S RNA. The volume of the DNA fraction was reduced to about 100-500 µl by evaporation in a vacuum desiccator. In different experiments 50-150 µl of avidin-spheres (10-15 mg/ml) were added either during or after the evaporation. The solution was adjusted to 1 M NaCl and allowed to stand for 12-16 hours at room temperature or 48 hrs at 0°C. Spheres and DNA bound to spheres were separated from free DNA by banding in CsCl as described (4) except that centrifugation was performed for 48 hrs. The amounts of DNA in the different fractions were determined by ^{3}H counting. DNA was released from the spheres and the RNA hydrolyzed by treatment with 0.2 M NaOH at 100°C for 20 min (14) or at 37°C for 16 hours. The 5S gene content of the several fractions was determined as described above.

RESULTS AND DISCUSSION

Preparation and Purification of RNA-cytochrome-c-biotin. tRNA or 5S RNA was reacted with cytochrome-c as described. The first step in the purification of the reaction product from the starting materials is passage over a CMC column in 0.01 M NaP buffer. Spectrophotometric monitoring showed that neither tRNA nor tRNA-cytrochrome-c binds to the column whereas the free cytrochrome-c does. The crucial step in the purification of RNA-cytochrome-c from unreacted RNA is HAP chromatography. As shown in fig. 1, tRNA elutes from HAP in a 0.15 M NaP wash, whereas RNA-cytochrome-c is eluted by 0.3 M NaP. The absorbance peaks at 410 and 260 mµ show that the material being eluted at 0.3 M NaP is the conjugate with 1:1 molar ratio.

Several comments should be made about the procedure. By using a 10-15 fold excess of cytochrome-c to RNA we decrease the probability of forming

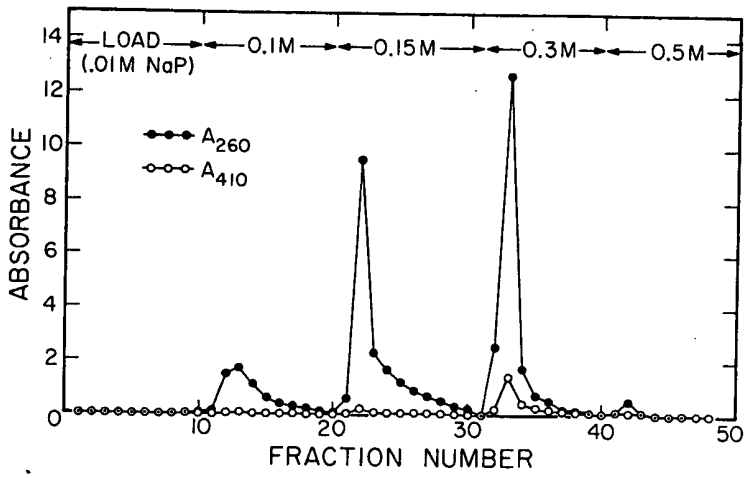


Fig. 1. Elution profile of tRNA-cytochrome-c from a HAP column. Batchwise elution of the reaction components was performed as described in Methods. tRNA and cytochrome-c concentrations were measured by absorbance at 260 mμ and 410 mμ, respectively. The column was loaded in 0.01 M NaP, pH 6.8. No A_{260} or A_{410} was observed in the washes with this solvent. The concentrations of tRNA and of cytochrome-c in the elution peaks were calculated as (tRNA) = $(A_{260}/5.4 \times 10^5)$ mole liter^{-1} and (cyt-c) = $(A_{410}/9.6 \times 10^4)$ mole liter^{-1}. The total amounts of tRNA and cytochrome-c in the peak eluting at 0.3 M NaP above are 35 and 34 nmoles, respectively.

molecules such as $(RNA)_2$-cytochrome-c. The elution profiles vary somewhat with the batch of HAP used and with its preparation. Our results have been reasonably reproducible when the HAP is freshly prepared for each experiment as described in Materials and Methods. Nevertheless it is necessary to monitor the absorbance profiles for each new purification, and to make slight adjustments of the salt concentrations of the elution buffers accordingly.

The final results of all experiments on the preparation of RNA-cytochrome-c and of RNA-cc-biotin are given in table I. The yields of RNA-cytochrome-c in some of the earlier experiments were poor. We now suspect that the Drosophila tRNA used was charged with amino acids, but we failed to include a deacylation step, hence the poor yield in these experiments. The best yields with deacylated E. coli tRNA are approximately 50%. For 5S RNA it proved necessary to pretreat the cytochrome with iodoacetate to inactivate RNAase, otherwise the 5S RNA was extensively degraded. We believe that this step is advisable for tRNA preparations also, but it was not done in the experiments of table I. Heating the RNA sample to 80° before coupling appears to improve the yield, possibly because it causes dissociation of aggregates formed during lyophilization or ethanol precipitation.

Table I

Effect of Different Treatments on Yields of RNA-cytochrome-c-biotin

RNA	Treatment	% Yield (1:1 RNA/cyto-c)	Molar excess of biotin/RNA	Number of biotins/RNA-cyto-c
E. coli tRNA	no heat step	28.7	50	5
E. coli tRNA	no heat step	12.7	33	3
E. coli tRNA	80°C, 1 min	46	190	7
E. coli tRNA	80°C, 1 min	48	100	7
[a]Dm tRNA	80°C, 1 min	11.2	240	9
Dm tRNA	80°C, 1 min	18.7	100	10
[b]E. coli 5S rRNA	no heat step	30	100	7
E. coli 5S rRNA	70°C, 10 min	55	100	5
Dm 5SrRNA	70°C, 10 min	56	100	9
Dm 5S rRNA	70°C, 10 min	43	100	5

[a]As mentioned in the text, Dm tRNA was not deacylated and hence lower yields.

[b]Results with 5S rRNA are those where cytochrome-c was pretreated with iodoacetate as described in Methods. Initially, when the iodoacetate step was omitted, the yields of the final product were low (0-5%).

As shown in table I, a molar excess of 50-100 fold of NHS-biotin to tRNA-cytochrome-c was used in order to obtain a final product with 3-10 biotins per cytochrome. Control experiments with unconjugated RNA gave undetectable binding of biotin after treatment with NHS-biotin and dialysis.

Cytrochrome-c is positively charged and does not elute from the negatively charged resin, CMC, until the NaCl concentration is raised to approximately 5 M, whereas both tRNA and tRNA-cytochrome-c are negatively charged and do not bind to the column even in 0.01 M NaP. The crucial step in the purification is the HAP chromatography step. Unligated cytrochrome-c elutes with approximately 0.5 M NaP, free tRNA with about 0.15 M NaP, and the 1 to 1 RNA-cytochrome conjugate at 0.3 M NaP. Several other separation methods were tried without success, including DEAE chromatography both in denaturing and nondenaturing conditions, CsCl centrifugation and gel filtration.

EM Mapping: The $\phi 80 psu_3^-/\phi 80$ heteroduplex. This heteroduplex is a convenient test system for tRNA mapping techniques. As shown in previous studies (15, 16) and sketched in fig. 2, the heteroduplex loop consists of a 3100 nucleotide segment of E. coli DNA and a 2100 nucleotide single-strand

segment of ϕ80 DNA. The substitution begins at the att site of ϕ80 DNA. The E. coli single strand segment contains 1 $tRNA^{tyr}$ gene at a position 1100 nucleotides from the att junction. Electron micrographs of two heteroduplexes labeled with spheres-avidin are shown in fig. 2. Micrographs (not shown) of heteroduplexes labeled with ferritin-avidin are comparable in appearance to those obtained by other methods (2, 15). A histogram of the spheres positions is given in Fig. 3. The results are in accordance with previous mapping data.

A considerable background of free spheres is evident in the micrograph. We have not found a procedure for separating unbound spheres from those attached to DNA, comparable to the sodium iothalamate buoyant banding procedure (1) that can be used to separate free ferritin from ferritin

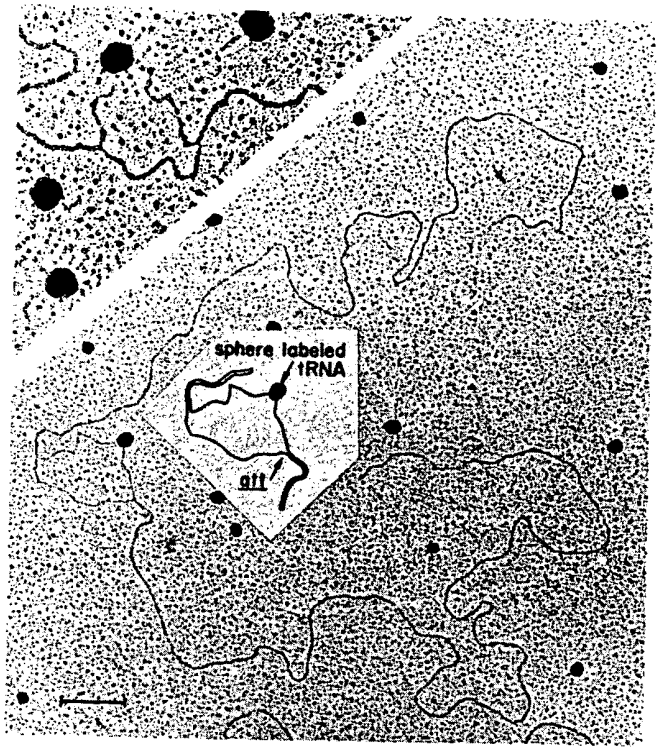


Fig. 2. Electron micrographs of sphere labeled tRNA genes on ϕ80/ϕ80psu$_3^-$ heteroduplex. One complete molecule is shown, with an inset sketch of the heteroduplex loop. The att site and the fork at the other end of the substitution loop are 23.8 and 19.3 kb from the left and right ends of the heteroduplex, respectively (15); therefore they are readily distinguished. An inset photograph of the heteroduplex loop of a second molecule is shown; the magnification is 2X that of the other photo. Bar = 1 kb.

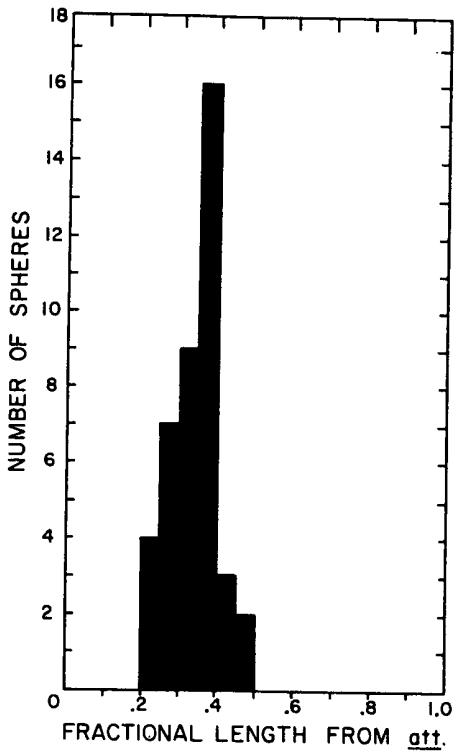


Fig. 3. Histogram of map position of $tRNA^{tyr}$ gene on the single strand of bacterial DNA of the $\phi 80/\phi 80$ psu_3^- heteroduplex. The horizontal coordinate is the fractional distance from the att site to the label. The measured position of the label is 1100 ($\pm$ 66) nucleotides from the att site.

labeled heteroduplexes. It may be noted however, that the spheres in the background seem to avoid the DNA strands.

By counting random fields we estimate labeling efficiencies per gene of 50-60% for both ferritin-avidin and the sphere-avidin labels using the cytochrome-c bridge. In experiments with the pentane diamine linker, the labeling efficiency with spheres was negligibly small (N.D. Hershey, personal communication). Presumably the greater length of the cytochrome-c linker is responsible for the efficient labeling in the present method. Nevertheless it should be noted that the efficiency of labeling either with spheres-avidin or ferritin-avidin is at best about 60%. The reason why a figure closer to a 100% is

not achieved is not known.

EM Mapping. Drosophila 4S and 5S genes. Studies from this laboratory on the mapping of 4S RNA genes on a 9.3 kb segment of Drosophila melanogaster (Dm) DNA contained on the recombinant DNA plasmid pCIT12 have been reported (7). The mapping data were obtained by EM mapping, using the technique described in detail in the present paper with a ferritin-avidin label, and by restriction endonuclease - hybridization mapping.

Electron micrographs from additional mapping experiments with the avidin-sphere label on the plasmid pCIT12 are shown in fig. 4. A histogram of the observed positions of the tRNA genes on the Dm insert is shown in fig. 5. In the previous study as well as in the present one labeled sites (genes) were found at the 3 positions 1.38 ± 11, 4.59 ± 0.17 and 8.38 ± 0.26 kb from the defined left end of the Dm insert. An additional gene mapping in the position 5.6 - 6.2 kb was found by restriction endonuclease mapping but was not detected at an appreciable frequency in the ferritin-avidin studies. The histogram in fig. 5 shows that this gene, mapped at 5.89 ± 0.36 kb was labeled at a frequency lower than that for the other genes but at a clearly detectable level in the present sphere-avidin studies. We do not know at present whether this improved labeling efficiency is due to the fact that the avidin spheres contain more avidins per label (8-10) than do the ferritin labels (1-2), or due to some other unknown factor.

Ferritin-avidin mapping studies, using the present techniques, on the recombinant plasmid pCIT9 with an insert carrying 3 Dm 5S RNA genes have been reported (6). Drosophila 5S genes are tandemly repeated with a regular spacing of about 380 nucleotides. Our labels -- ferritin-avidin or spheres-avidin-- are multivalent, in that they contain several biotin binding sites per label. Many tangled structures were seen because one label was attached to several hybridized RNA-biotin molecules along a DNA strand. Such molecules cannot be accurately analyzed; nevertheless we have estimated that the overall efficiency of labeling per gene is 40-50% in the various experiments.

As an overall evaluation then, the present method gives 40-60% labeling efficiency in practical problems. Resolution is probably limited by the diameters of the labels - about 200 Å for ferritin-avidin and 600 Å for spheres-avidin. At present, difficulties are encountered with closely spaced genes because of the multivalent character of the labels. Further work is needed to develop a ferritin-avidin conjugate with only one avidin per ferritin and with a high efficiency of labeling, so that closely spaced multiple genes can be mapped.

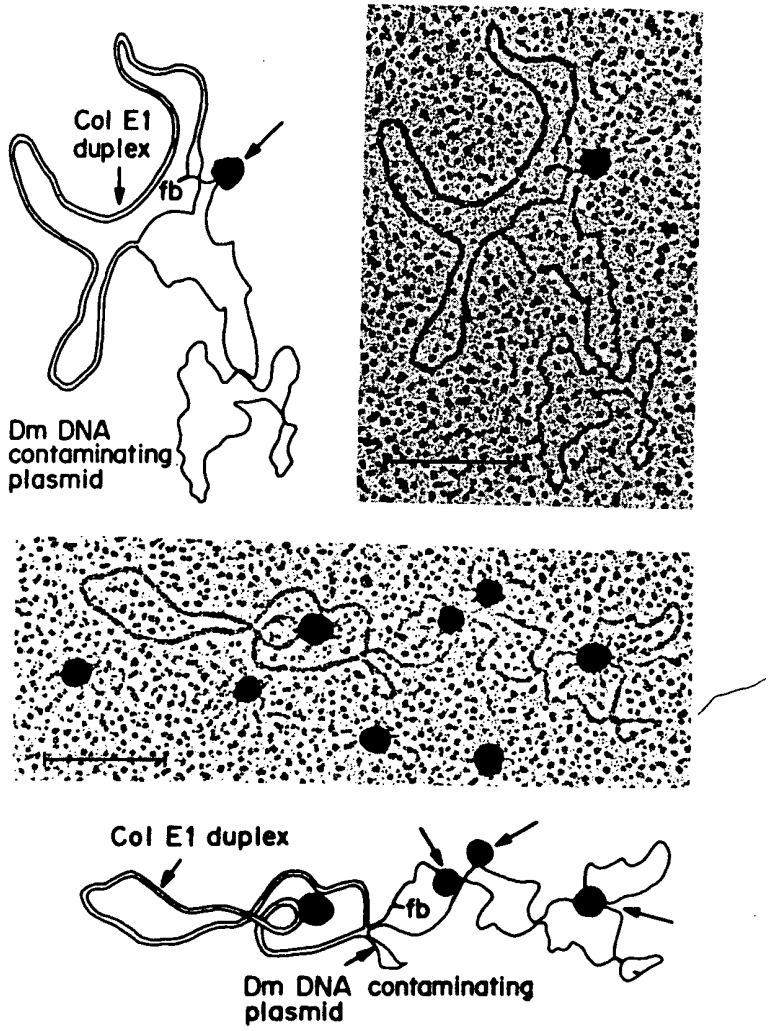


Fig. 4. Electron micrographs of sphere labeled tRNA genes on a single strand of the recombinant plamsid pCIT12. For a full explanation of the structures, see (7). Briefly, the molecules shown are heteroduplexes of pCIT12, which is ColE1 fused to a 9 kb Dm insert, with a second contaminant plasmid consisting of ColE1 fused to another short Dm insert. A secondary structure feature, fb, on the 9 kb single strand serves to orient that strand. The single tRNA gene labeled in the upper micrograph is at the position 8.38 $\pm$ 0.26 in fig. 5; the three sphere labels in the lower micrograph are at the positions 1.38 $\pm$ 0.11, 5.80 $\pm$ 0.36 and 8.38 $\pm$ 0.26 in fig. 5. In addition there is a sphere nonspecifically attached to the ColE1 duplex. These are rare. Bars = 1 kb.

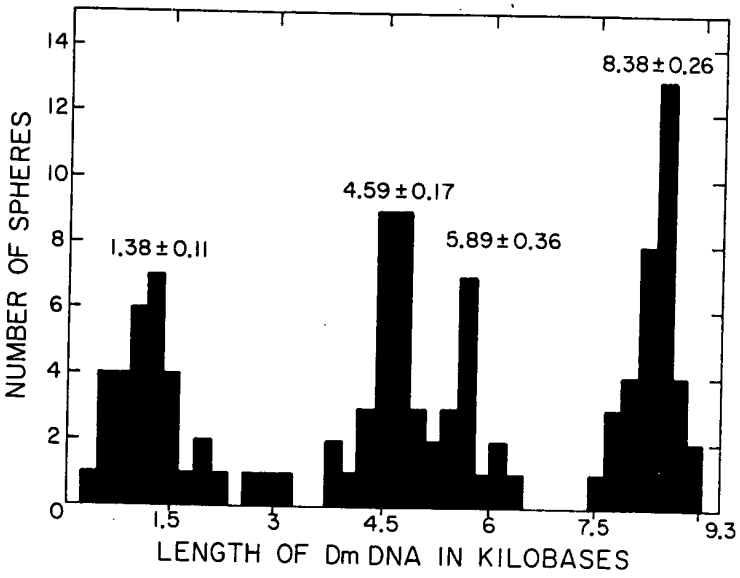


Fig. 5. Histogram of the distribution of Dm tRNA gene positions on the pCIT12 plasmid. The measurements were done as described (7). In addition to the 3 genes found in the previous ferritin mapping study, a 4th gene at 5.89 kb was labeled here, although to a lesser extent. Its position is in agreement with that determined for the 4th gene by the restriction endonuclease mapping.

Gene Enrichment. In these experiments, high molecular weight Drosophila DNA is incubated with 5S RNA-cc-biotin. Hybridization is carried out in a high formamide solvent under conditions where RNA:DNA association is favored over DNA:DNA association (13). Those DNA strands which hybridize to the 5S RNA are separated from all other strands by reaction with avidin-spheres and buoyant banding. These experiments are basically identical to those previously described for the enrichment of the rRNA genes of Drosophila (4), except that, as appropriate for the short length of the 5S RNA, we use a terminal cytochrome-c bridge between the RNA and the biotin instead of random crosslinking of cytochrome-biotin to the RNA by CH_2O.

There are 160 5S RNA genes of Drosophila (17). They are tandemly repeated with a repeat spacing of about 380 nucleotides. They may occur as two clusters of approximately 80 genes each or there may be a single cluster of length 63.4 kb containing all of the genes, as suggested by restriction digest studies of the chromosomal DNA (17, 18). All told, the genes plus spacers constitute 1.73×10^{-4} of the haploid Dm genome (1.8×10^8 base

pairs). In the present experiments the bulk of the single strands of Dm DNA ranged in length from 10-100 kb. Thus the strands carrying 5S genes would have 25 or more 5S RNA-cc-biotin molecules hybridized. (There is every theoretical reason to expect that the efficiency of RNA:DNA hybridization is almost 100%.) For a strand of length 10 kb with one sphere attached the weight fraction of sphere mass is 0.96. If several spheres are attached per 10 kb the weight fraction is still closer to 1. Thus by the rules formulated by Pellegrini et al. (5) the buoyant density of the DNA sphere complex would be close to that, 1.25 g/ml, of uncomplexed spheres. The efficiency of labeling in the electron microscope experiments was 50% per gene. Just how many avidin-spheres would be bound to a 10 kb strand carrying 25 hybridized 5S RNA-cc-biotin molecules is uncertain because we do not know how many of these biotins would bind to a single avidin-sphere rather than to different avidin-spheres.

The results of several gene enrichment experiments are presented in table II. In all three experiments, almost all (greater than 88%) of the 5S genes were recovered in the enriched fraction. In the first experiment, about 1.5×10^{-2} of the total DNA was in the enriched fraction whereas in the second and third experiments only about 6.5×10^{-4} of the DNA was in the

Table II

Results of Enrichment for 5S rDNA from Total Drosophila DNA

	% Total DNA in		% 5S rDNA in		Enrichment Factor
	Enriched	Depleted	Enriched	Depleted	
Theoretical Experiment	0.0173	99.923	100	0	5780
1	1.48	98.52	99.9	not detected	67
2	0.067	99.933	91.5	8.5	1351
3	0.063	99.937	88.8	9.8	1411

The volume of the DNA after the Sepharose 2B step is usually 7-10 ml. The salt concentration is 0.1 M. This volume is reduced by evaporation by a factor of 10 and the salt concentration raised to 1.0 M for sphere labeling under conditions of minimal electrostatic interaction between the positive avidins on the spheres and the negative DNA. In the first experiment, the spheres were added before the evaporation step. Perhaps this caused more quasi-irreversible electrostatic binding between spheres and DNA. In experiments 2 and 3, spheres were added after evaporation. In the 3rd experiment, labeling was done at 0° for 48 hr instead of at room temperature for 16-24 hours.

enriched fraction. Therefore in the first experiment the 5S DNA after one cycle of enrichment was about 1% pure, whereas in the second and third experiments it was about 25% pure, In control experiments where no RNA was added approximately 3.4×10^{-4} of the DNA was found in the sphere band. Therefore of the total of about 6.5×10^{-4} DNA in experiments 2 and 3, about 50% may be attributed to general binding or trapping of DNA by the spheres, 25% to specific gene enrichment, and 25% to other causes, possibly formation of networks or partially duplex DNA with a strand bearing a 5S gene (see below).

The two major technical differences between experiment 1 and the more successful experiments 2 and 3 were:

1) In the latter experiments, the excess of unlabeled (no cytochrome-biotin) rRNA (which included 5.8S rRNA as well as 18 and 26S) added was 100 times the amount of 5S RNA, whereas in the first experiment there was only a 15 fold excess. Whereas 5S genes plus spacers make up 1.7×10^{-4} of Dm DNA, rRNA genes plus spacers constitute 6.4×10^{-3}; therefore it is necessary to completely compete out hybridization of any biotin labeled rRNA fragments present as contaminants in the 5S preparation with the cold rRNA. In their filter hybridization experiments, Tartof and Perry (19) found that it was necessary to use a 100 fold excess of unlabeled rRNA in order to accurately assay for the number of 5S RNA genes.

2) As explained in a footnote to table II, there was a difference in a concentration step of the sphere-avidin-DNA mixture between experiments 1 and experiments 2 and 3 which may have decreased the amount of non-specific binding in the latter.

A small fraction of the sphere band from the CsCl gradient was directly diluted into formamide solution and spread for electron microscopy. The DNA structures observed were of the following types: a) Single strands with one or many spheres. Strands with many spheres were tangled and condensed around the spheres, as expected in view of the close spacing of the genes and the several avidins attached to each sphere. b) Single strands with no spheres attached. These were presumably released from the spheres by breakage. c) Molecules that were partially duplex, due to some DNA:DNA reassociation. d) Networks of single strands, perhaps due to tangling of the long strands in the high salt medium. Factors (c) and (d) may contribute to the amount of non-coding strands in the enriched fraction. The DNA strands observed had about the same length distribution as the input DNA, showing that the gene enrichment procedure does not cause much chain breakage.

Further discussion. In several test systems, the efficiency of labeling with the cytochrome-c-biotin attached to the 3' terminus of 4S and 5S RNA was 50% with either avidin-spheres or ferritin-avidin. The efficiency of labeling by spheres-avidin for cytochrome-biotin randomly crosslinked to RNA with CH_2O is reported to be sustantially lower than this figure (4). The present method could be applied to genes for long RNA's as well as for short ones. For long RNA, it would be advantageous to degrade the RNA to a length of 100-400 nucleotides, and expose new 2', 3' OH ends with alkaline phosphatase before coupling to cytochrome-c. Thus one would provide several cytochrome-biotin affinity labels per gene. In general then, the present technique appears to be a very useful addition to methods of gene enrichment and electron microscope gene mapping.

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Bij voorbaat hartelijk dank.

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An Electron Microscope Study of the Relative Positions of the 4S and Ribosomal RNA Genes in HeLa Cell Mitochondrial DNA

Lynne Angerer and Norman Davidson
Division of Chemistry
William Murphy, Dennis Lynch, and
Giuseppe Attardi
Division of Biology
California Institute of Technology
Pasadena, California 91125

Summary

The 4S RNA genes in HeLa mitochondrial DNA (mtDNA) have been mapped by electron microscopy using the electron-opaque label ferritin. This method is based on the high affinity interaction between the protein, avidin, and biotin. 4S RNA, covalently coupled to biotin, was hybridized to single-stranded mtDNA. The hybrids were then labeled with ferritin-avidin conjugates. The positions of ferritin-labeled 4S RNA genes were determined relative to the rRNA genes on both heavy (H) and light (L) strands of mtDNA. This region was recognized as a duplex segment after hybridization either with rRNA in the case of H strands or with DNA complementary to rRNA in the case of L strands.

Our studies suggest that at least nineteen 4S RNA genes are present in the HeLa mitochondrial genome. On the H strand, we have confirmed the nine map positions found in a previous electron microscope mapping study (Wu et al., 1972) and obtained evidence for three additional 4S RNA genes. On the L strand, seven 4S RNA genes have been mapped. The nineteen genes are distributed more or less uniformly around the genome. There is a pair of closely spaced genes, approximately 150 nucleotides apart, on the H strand, and another closely spaced pair on the L strand.

Introduction

In previous studies, eleven to twelve genes coding for 4S RNA were found in the HeLa mitochondrial genome by filter saturation hybridization experiments (Aloni and Attardi, 1971a) and by electron microscopic mapping studies with ferritin-labeled 4S RNA (Wu et al., 1972). Eight genes were estimated to be on the heavy (H) strand by hybridization saturation with labeled tRNA, while nine genes were identified by physical mapping; both techniques indicated the presence of three genes on the light (L) strand. This limited coding capacity suggested either that the proteins synthesized in the mitochondrion were restricted with respect to amino acid composition, or that some tRNA species were imported into the mitochondrion from the cytoplasm. In support of the first possibility, it was found that chloramphenicol-sensitive amino acid incorporation by HeLa mitochondria was very low for eight amino acids, including most of the charged polar ones (Costantino and Attardi, 1973).

To determine whether the amino acid specificities of tRNAs coded by mitochondrial DNA (mtDNA) were in agreement with the chloramphenicol-sensitive amino acid incorporation data, Lynch and Attardi (1976) have recently measured the hybridization of tRNAs acylated with radioactively labeled amino acids to separated strands of mtDNA. Their studies showed that there are at least twelve genes on the H strand and five on the L strand coding for tRNAs of sixteen different amino acid specificities. While this is a larger number of tRNA genes than detected previously in HeLa mtDNA, we believe it possible that it still represents a minimum estimate for several reasons. First, multiple identical genes or genes coding for isoaccepting tRNAs on the same DNA strand would not be detected. Second, some tRNA species may not be aminoacylated efficiently by mitochondrial synthetase preparations under the conditions used, so that their hybridization would not be detected. Third, tRNAs present in very low concentration might be missed by this approach since these hybridizations are done in DNA excess.

Since at least five additional genes coding for tRNA were found in the HeLa mitochondrial genome, we decided to reinvestigate the arrangement of 4S RNA genes by using a newly developed method of labeling hybridized RNA with ferritin for direct electron microscopic examination (T. R. Broker, L. Angerer, N. D. Hershey, P. Yen, M. Pellegrini, and N. Davidson, manuscript in preparation). In this procedure, a small molecule, biotin, is covalently coupled to the oxidized 3' terminus of RNA molecules. The RNA is then hybridized to a DNA strand. The position of the RNA:DNA hybrid on the DNA strand is mapped by incubating with a covalent conjugate of the electron-opaque label ferritin with the protein avidin. The labeling reaction is based on the rapid and strong noncovalent association of avidin with biotin (Green, 1963).

The primary purpose of the present studies was to determine the number and distribution of 4S RNA genes on the L strand by mapping ferritin positions relative to the ribosomal RNA gene positions. These genes were identified as a double-stranded region after hybridization either with DNA complementary to 12S and 16S rRNA (rDNA) or with a unique DNA fragment purified from a HpA II restriction endonuclease digest of HeLa mtDNA. The restriction fragment contains major portions of the genes coding for 12S and 16S rRNA. Similarly, 4S RNA genes on the H strand were mapped relative to the rRNA-DNA hybrid region. Evidence is presented which confirms the positions of the nine H strand 4S RNA genes and three L strand genes mapped previously

in our laboratory (Wu et al., 1972). In addition, four other 4S RNA genes have been mapped on the L strand, and evidence has been obtained suggesting that three additional genes may be located on the H strand of HeLa mtDNA. It should also be noted that Heitzmann and Richards (1974) independently conceived of the use of the avidin-biotin interaction for labeling purposes, and have developed a technique for ferritin-labeling membrane components.

Results

Properties of the Reference Markers

The length distribution, as determined by electron microscopy, of the 12S and 16S rDNA, prepared as described in Experimental Procedures, was very heterogeneous. The number average lengths of the 12S and 16S rDNA:rRNA duplexes were 645 (±280, 43%) and 717 (±348, 48%) nucleotide pairs, respectively. The length distributions of the single-strand DNA after denaturation in hot NaOH (0.1M, 60°C, 4 min) were similar. Thus these fragments have average lengths of about 63% and 45% of the full lengths (Robberson et al., 1971) of the respective genes. Presumably, fragmentation occurred during the S1 nuclease digestion. The 12S DNA preparation contained some molecules longer than 12S rRNA, probably from cross-contamination of 12S with 16S rRNA, but it is not possible to estimate the extent of cross-contamination.

In contrast, the other marker for the rRNA gene region, which was a fragment isolated from a HpaII restriction endonuclease digest of HeLa mtDNA, was fairly homogeneous in length. The duplex- and single-strand lengths were 1860 (±250, 13%) and 1860 (±160, 9%) nucleotides (11.9% of the genome), indicating no single-strand nicks in the duplex fragment. This DNA fragment contains sequences complementary to a major protion of both 12S and 16S rRNA as determined by RNA:DNA hybridization experiments utilizing RNA species purified from isolated mitochondrial ribosomal subunits (D. Ojala and G. Attardi, unpublished results).

L Strand Mapping

In the first experiments, purified HeLa mitochondrial L strand DNA, preannealed to remove any trace of H strand contaminants, was hybridized with 12S and 16S rDNA and biotin-4S RNA as described in Experimental Procedures. The results of this experiment were compiled in the histogram shown in Figure 1a. In measurements on 142 molecules, four distinct sharp peaks (labeled L1, L2-L3, L4, and L5) and a fifth broader peak (labeled L6 and L7) were resolved relative to 12S and 16S rDNA duplex regions. Furthermore, the second peak which is nearly 2 times larger than any other peak, represents two distinct map positions (L2, L3) which are too closely spaced to be resolved in the histogram: on a number of individual molecules, two ferritin molecules separated by approximately 150 ± 40 nucleotides were mapped at the position of this peak.

It should be noted that in almost every case it was not possible to distinguish the spacer region between 12S and 16S rDNA: L strand hybrids, and therefore the duplex region was scored as a con-

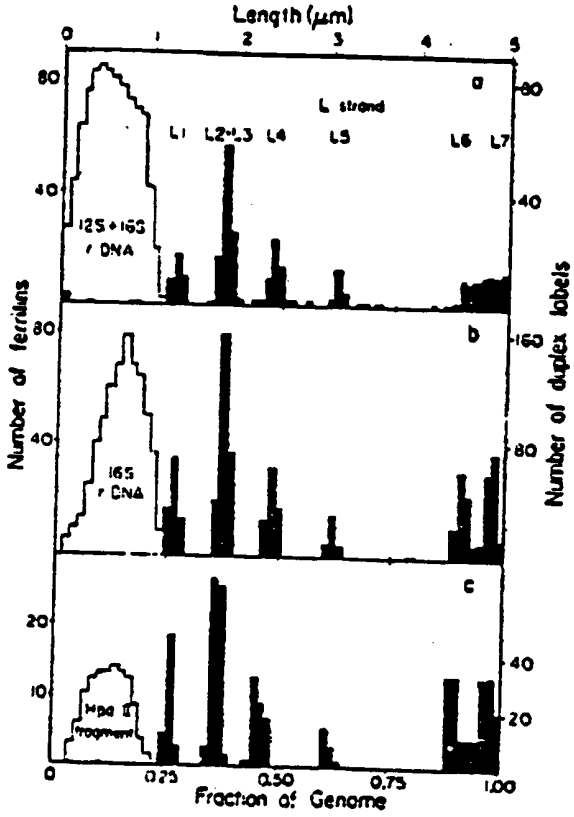
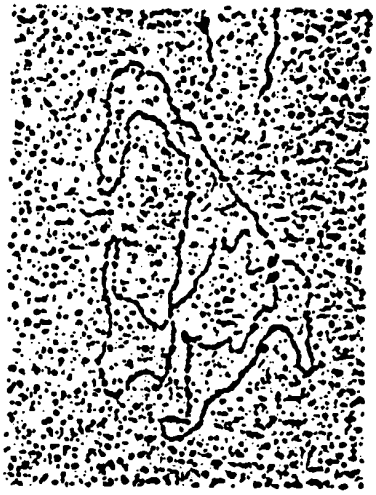


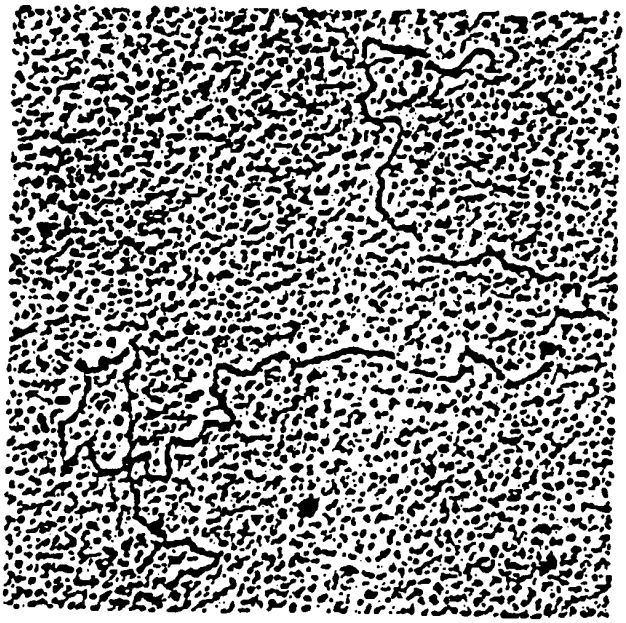
Figure 1. Histogram of 4S RNA Map Positions on the L Strand of HeLa mtDNA Relative to the Positions of Reference Markers Located in the rRNA gene Region.

(a) Relative to 12S + 16S rDNA; (b) relative to 16S rDNA; (c) relative to HpA II fragment 3. The peaks are numbered from L1 to L7. Peak L2-L3 includes two closely spaced 4S RNA genes. The data in the histograms were compiled from measurements on both circles (approximately 50%) and linears (approximately 50%) whose lengths were very close to that of circles on the same grid. The lengths of all these molecules were taken as equivalent to one genome. Since the reference markers did not allow us to select one of the two possible orientations for each molecule, the molecules were aligned such that the ferritin labels fell into the fewest number of discrete groups while maximizing the extent of overlap of the duplex marker regions. For comparison with previous mapping studies (Wu et al. 1972), a scale in units of microns is also given where 5 μ equals one genome length. The length interval used to construct the histogram was 0.11 μm.

tinuous sequence. One possible reason for this is that since the rDNA was fragmented as described above, the thickness of the duplex regions was variable due to small single-stranded segments in the hybrid.

To determine the orientation of 4S RNA sites relative to 12S and 16S ribosomal gene regions for comparison with the H strand map (Wu et al., 1972), the same experiment was repeated using only the 16S rDNA preparation as the marker. The results of measurements on 192 molecules are illustrated in the histogram shown in Figure 1b. The distribution of 16S rDNA: L strand hybrids is skewed to one side of the ribosomal gene region (see Figure 1a). Thus on a linear map constructed from these histograms, the 12S rRNA complementary region is to the left of the 16S gene region. Furthermore, in this experiment, the fifth peak is clearly resolved into two peaks of equal size, labeled L6 and L7 in Figure 1a. On a number of molecules, both sites were occupied, thus confirming the presence of two distinct 4S RNA sites. A compilation of the results obtained thus far indicated that seven sites were labeled with approximately equal efficiency except for peak L5. In both experiments, the frequency of labeling this gene was about 40% that of the other





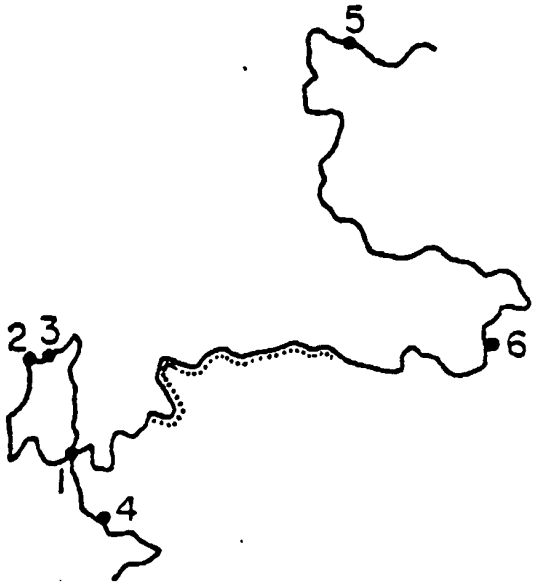


Figure 2. Electron Micrographs of L Strand mtDNA Hybrids with Hpa II Fragment 3 and 4S RNA-Biotin Labeled with Ferritin-Avidin
Explanatory tracings are given indicating the positions of the 4S RNA genes and reference duplex segment. An ambiguous duplex region is indicated by dotted lines.

sites. Based on the existence of seven different 4S RNA sites, an overall labeling efficiency of 35% was achieved in these experiments.

In the third experiment for mapping the 4S RNA sites on the L strand DNA, several important modifications were made in the procedure. The HpA II restriction fragment was used as the marker for the rRNA gene region. It provided a better reference segment than the 12S and 16S rDNA markers because the latter were somewhat broken. In particular, the end points of the duplex could be more accurately identified for the HpA II fragment. Second, another preparation of 4S RNA-biotin was used. In addition to purification on sucrose gradients, the 4S RNA population was passed through oligo(dT)-cellulose to remove any possible small poly(A)-containing RNA fragments: these, if present in sufficient abundance, might produce an artifactually high number of 4S RNA binding sites. However, less than 1% of the total 4S RNA preparation was bound to the oligo(dT)-column, suggesting that the 4S population used here does not contain a substantial amount of poly(A)-containing RNA. Third, the L strand hybridizations were carried out in a different annealing buffer composed of 40% formamide, 0.3 M NaCl, 0.1 M Tris, and 0.001 M EDTA (pH 8.0), instead of 40% formamide, 0.2 M sodium phosphate (pH 7.5), as used in the first two experiments reported here and by Wu et al. (1972) in previous HeLa mtDNA mapping experiments. We suspect, although we do not have decisive data, that this is an improved solvent for hybridization reactions. While the detection of duplex marker regions was still difficult in some cases, in general, there were fewer molecules whose interpretation was ambiguous. Several examples of ferritin-labeled L strands from this experiment are shown in Figure 2.

The positions of sites of ferritin labeling relative to sequences complementary to the HpA II restriction fragment are shown in the histogram in Figure 1c. The pattern is exactly the same as found in the previous two experiments. The length of the Hpa II fragment hybridized to L strands (1807 ± 159) nucleotides) is in good agreement with the duplex length of the restriction fragment (1860 ± 250 nucleotides). Its position on the histogram confirms that it contains sequences complementary to both 12S and 16S rRNA.

Although a detailed scoring of the overall gene labeling efficiency was not performed in this experiment, many more molecules with three or more ferritin labels were found, indicating that the efficiency was higher than the 35% observed in previous experiments. Only those molecules were photographed which had the marker and one or more ferritins. Of 68 such molecules photographed, 59 had three or more ferritins attached. The gene-labeling efficiency calculated from this sample is 45%. This is probably only a slight overestimate of the true labeling efficiency, since very few molecules in the population had no ferritin labels. It is not known whether the higher efficiency observed here can be attributed to the change in annealing conditions or to an improved 4S RNA-biotin preparation. It should be noted that despite an overall higher labeling efficiency, the frequency of labels at the position corresponding to L5 was still only 50% of that at other L strand sites.

H Strand Mapping

In previous physical mapping studies using ferritin-labeled 4S RNA from HeLa mitochondria (Wu et al., 1972), nine sites were detected on the H strand. Since the more recent data of Lynch and Attardi (1976) indicated that at least twelve tRNA genes were present on the H strand, the 4S RNA map of H strand was reinvestigated using the ferritin-avidin:biotin-4S RNA labeling technique. The hybridizations were carried out as described in Experimental Procedures, and the positions of ferritin labels relative to rRNA markers were determined on 182 molecules. A histogram of the data is shown in Figure 3. The presence of the nine sites previously mapped was confirmed, and evidence was ob-

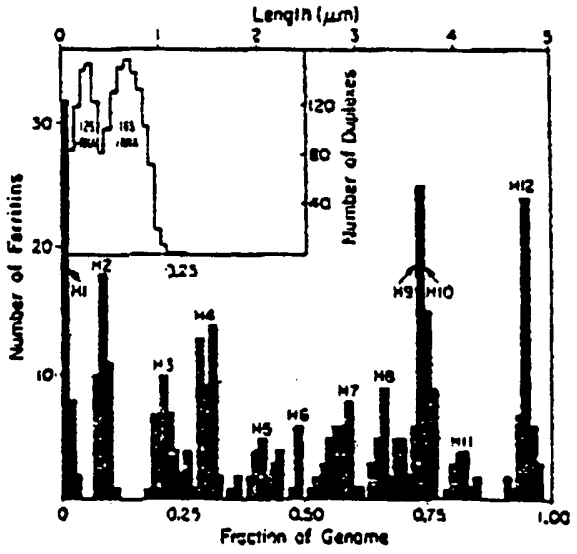


Figure 3. Histogram of 4S RNA Map Positions on the H Strand of HeLa mtDNA Relative to the Positions of rRNA-DNA Duplex Regions Shown in the Inset

The procedure used to compile the data in this histogram is described in the legend to Figure 1. In some cases, the spacer region between the 12S and 16S rRNA:DNA hybrids was sufficiently unambiguous to allow us to fix the orientation of the molecule. The sites are numbered from H1 to H12. The length interval used in constructing the histogram was 0.074 μm. Lengths are given both in μm (top) and fraction of the genome (bottom). Positions numbered H5, H6, and H11 represent 4S RNA genes not mapped in the previous electron microscope study (Wu et al., 1972).

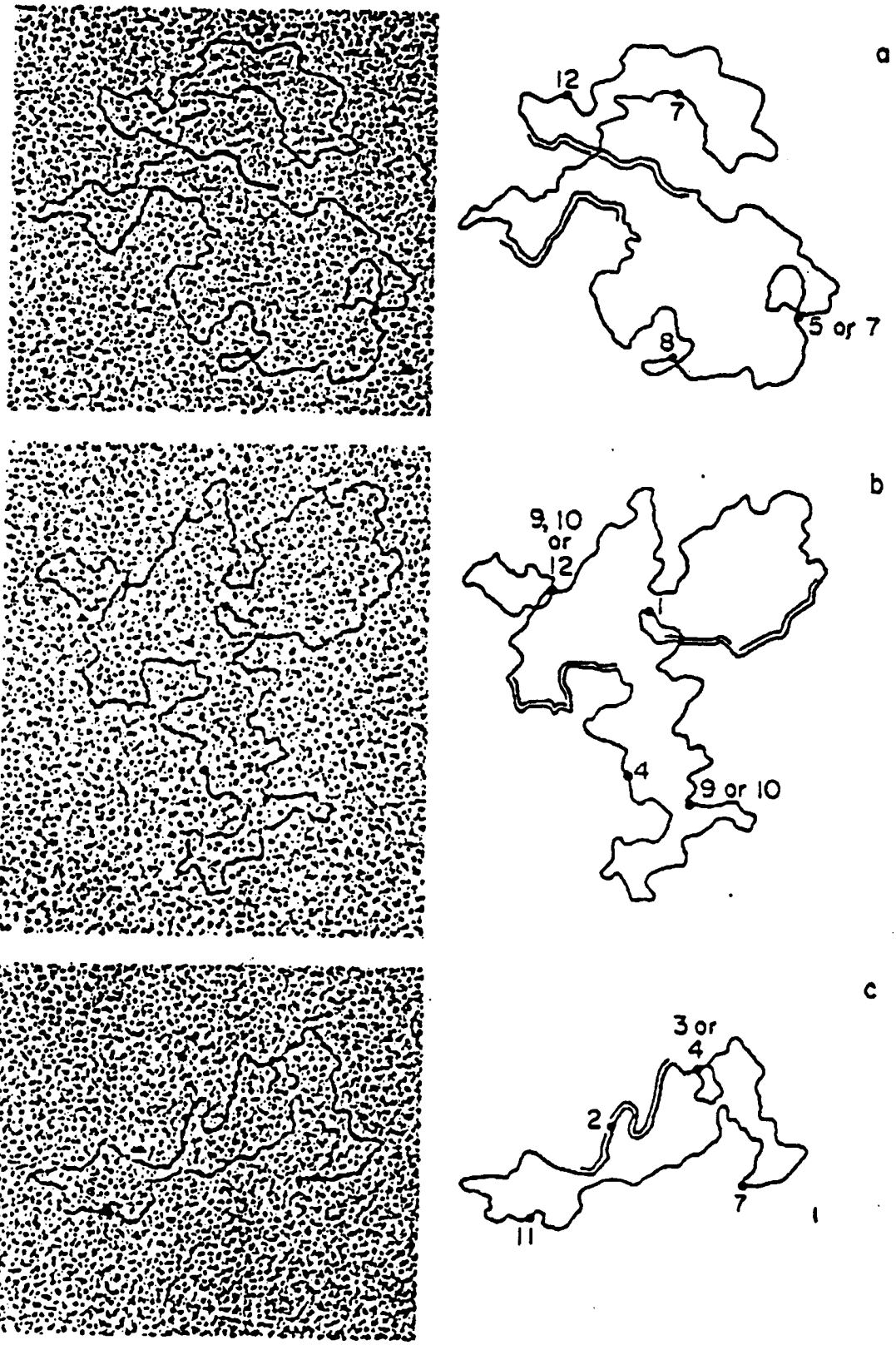


Figure 4. Electron Micrographs of H Strand mtDNA Hybrids with 12S and 16S rRNA and 4S RNA-Biotin Labeled with Ferritin-Avidin
Explanatory tracings are presented. The molecules shown in (a) and (b) constitute dimers. Note that identical sequences are diametrically opposed, confirming a head-to-tail arrangement of the monomer genomes. In (c), a monomer H strand is shown.

tained for the existence of at least three additional sites, denoted by the numbers H5, H6, and H11 in Figure 3. While the frequency of labeling at these additional sites is not as high as at other sites, it is significantly greater than that expected for nonspecific background binding.

Several examples of ferritin-labeled H strands are shown in Figure 4. In this preparation of mtDNA, about 30% of the molecules were dimers with both the rRNA:DNA duplex regions and with some of the ferritin labels diametrically opposed. This indicates a head-to-tail arrangement of the monomer genomes in the dimers, as previously shown for the mtDNA dimers from human leukemic leukocytes (Clayton and Smith, 1975). Dimer circles containing rRNA hybrids and several ferritin labels are shown in Figures 4a and 4b.

The gene-labeling efficiency in this experiment was quite low, between 10 and 15%, based on twelve H strand 4S RNA sites. It is not known whether this was due to the stringent hybridization conditions used to minimize DNA-DNA reassociation, to a deterioration of the 4S RNA-biotin, which had been stored at −70°C for 6 months, or to the fact that under the hybridization conditions used (C_0t = 15 × 10^{-2} moles sec l^{-1} or 15 × $C_0t_{1/2}$ for total 4S RNA), some 4S RNA-biotin species may have been in limiting concentration. With respect to the last possibility, it should be noted that the effective concentration of an individual 4S RNA species in these experiments depends not only on its representation in the original 4S RNA population, but also on its recovery through the biotin derivatization procedures (see Discussion).

Discussion

The final results of our electron microscopic mapping study are given in Figure 5. Seven 4S RNA sites have been localized on the L strand and twelve on the H strand, giving a total of 19 sites in the genome.

Technical Considerations

In these experiments, we have applied a new technique for visualizing these genes in the electron microscope in an effort to improve our gene-labeling efficiencies. In previous physical mapping studies, such sequences were recognized by hybridizing 4S RNA covalently attached to the large iron-containing protein, ferritin (molecular weight = 9 × 10^5) (Wu et al., 1972). This procedure has several potential disadvantages. First, it was suggested that ferritin might interfere with the hybridization of attached 4S RNA, resulting in suboptimal gene-labeling efficiencies (approximately 50%). Second, the structure of ferritin and, in particular, that of bromoacetylated ferritin, is sensitive to the relatively high concentrations of formamide (40%) used for hybridization. To circumvent these difficulties, we attached a much smaller molecule, biotin (molecular weight = 244), to 4S RNA, which should not significantly reduce the hybridizability of the RNA. After hybridization formamide was removed, and the 4S RNA-DNA hybrid was treated with ferritin-avidin under mild conditions which do not affect ferritin structure, that is, the protein does not denature and precipitate from solution, and appears as a dark round spot in the electron microscope.

Despite these improvements, the gene-labeling efficiences obtained here are still only 40–50%, comparable to those observed by Wu and Davidson (1973). However, in experiments performed by a number of investigators in our laboratory, the RNA-biotin:avidin-ferritin technique has proved to be much more reproducible in giving at least partial labeling of 4S RNA genes. The reasons for suboptimal labeling efficiences are still not understood. As indicated in Experimental Procedures, it seems improbable that the concentration of 4S RNA and the time of incubation under hybridization conditions (C_0t) were insufficient to allow saturation of available 4S RNA coding sequences. In the L strand mapping experiments, the C_0t with respect to total 4S RNA is estimated to be 120 times the theoretical $C_0t_{1/2}$. Even if an individual 4S RNA species is

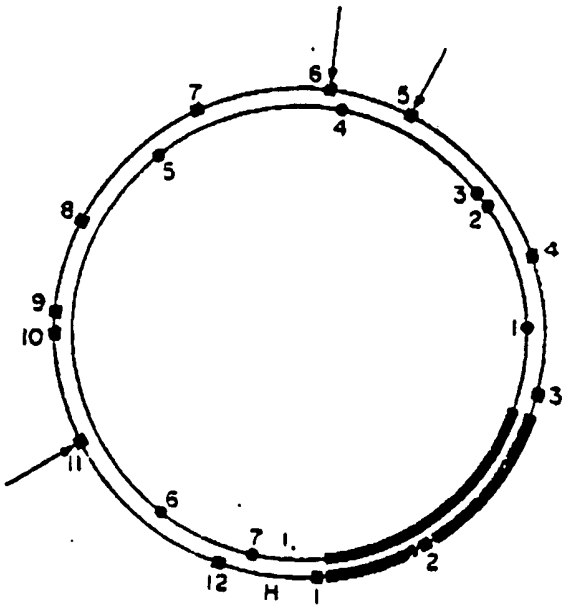


Figure 5. Combined Circular Map of 4S RNA Map Positions on the H and L Strands of mtDNA Obtained from the Histograms Shown in Figures 1 and 3

The rRNA gene region is denoted by the heavy black lines. The arrows mark those positions on the H strand which were not mapped in the previous study (Wu et al., 1972).

present in the total population 10 times less frequently than other 4S RNA species, we would predict that well over 90% of its coding sequences should be in hybrid. In the H strand mapping experiment, the 4S RNA C_0t achieved is not as easy to estimate, since RNA:DNA hybridization rates under the more stringent conditions used are not as well characterized. However, preliminary data from our laboratory suggest that the rates should be at least as fast, if not faster (J. Casey, personal communication); we therefore estimate that the C_0t was at least 15×10^{-3} moles sec l^{-1} or 25 times the theoretical $C_0t_{1/2}$.

From the preceding argument, it seems improbable that low labeling efficiencies are due to failure to saturate the 4S RNA coding sequences. This inference is supported by the observation that we routinely observe about 50% labeling efficiency of the ϕ80 *psu3*$^+$/ϕ80 heteroduplex, with one $tRNA^{Trp}$ gene, that we use as our standard test system for tRNA gene labeling (T. R. Broker et al., manuscript in preparation).

There are several factors which could conceivably contribute to low labeling efficiences. First, during the process of attaching biotin to 4S RNA, several treatments are made which could select against certain tRNA species. In particular, the BH_4^- reduction step modifies 7-methyl guanosine and dihydrouridine residues so that the phosphodiester chains are sensitive to cleavage at these points (Wintermeyer and Zachau, 1975; Cerutti and Miller, 1987). Second, the presence of any free avidin or subunits of avidin in the preparation of ferritin-avidin could significantly reduce ferritin labeling if they are able to react with biotin at faster rates than do the ferritin-avidin conjugates. Although we have been unable to detect free avidin in our preparations even after storage at 4°C for several months, it is possible that some degradation occurs during the labeling incubation at room temperature. It is important to note here that very high concentrations of ferritin-avidin are required to achieve significant labeling (5000 fold higher concentration than the hybrid). This suggests that only a small fraction of ferritin-avidin conjugates is capable of labeling 4S RNA-biotin when it is hybridized to DNA.

The previous electron microscope mapping experiments on the 4S genes of HeLa mtDNA (Wu et al., 1972) indicated that there were nine sites on the H strand, while the presence of three sites on the L strand was inferred from measurements of interferritin distances. There are several possible reasons for the discrepancy between the results obtained from the two ferritin labeling studies. Since Wu and her colleagues did not have the advantage of mapping from a fixed reference point on the L strand, the number of genes they determined by measuring interferritin distances might have been an underestimate if there are multiple pairs of genes with similar spacings and if the gene labeling efficiency is low. The possibility that the composition of the 4S RNA population was different in the two studies remains, but is considered improbable. In mapping 4S RNA sites on H strand mtDNA, the additional three sites (possibly more) that were observed in this study occurred in relatively low frequency, approximately 30–50% that of some of the other sites. In this experiment, the hybridization conditions [80% formamide, 5 × SSC, 0.03 M Tris (pH 8.0), 46°C, 35 min] were significantly different from those used by Wu and her co-workers [40% formamide, 0.2 M phosphate buffer (pH 7.5), 37°C, 1 hr]. The more stringent hybridization conditions were chosen because they favor RNA-DNA hybridization over DNA-DNA reassociation (J. Casey, personal communication). In the present experiments, it was necessary to minimize the DNA-DNA reaction since we had to use unseparated strands of mtDNA. If the rate of hybridization of some 4S RNA species is too low to be detectable in 40% formamide, 0.3 M Na^+, but is significant in 80% formamide, 5 × SSC (0.98 Na^+), then at least part of the discrepancy might be explained.

It can be seen in Figure 5 that the L4 and H6 sites are very close to each other. The resolution of the map is not fine enough to determine whether or not these sites are in the same position. While we believe that the hybridization conditions are probably sufficiently stringent to eliminate nonspecific binding of a tRNA to the wrong strand by virtue of its self-complementary sequences, that possibility cannot be rigorously excluded.

Most of the sequences we label probably code for tRNA, but it is possible that some other small stable RNA species are present in the 4S RNA population. For example, Dubin, Jones, and Cleaves (1974) have suggested that hamster cell mitochondria contain a 3S RNA species which may be the mitochondrial equivalent of cytoplasmic 5S RNA. We believe it improbable that we detect labeling due to hybridization of mRNA fragments for several reasons. First, only those molecules with 3′ OH groups can be labeled because of the chemistry used for attaching biotin to RNA. Fragments of mRNA arising from endonuclease digestion should have primarily phosphate groups at their 3′ ends. Second, in one experiment, the 4S RNA preparation was passed through oligo(dT)-cellulose to remove any poly(A)-containing RNA fragments. This additional purification step did not alter the distribution of ferritin labels in the subsequent mapping experiment.

From the results of our studies, we conclude that there are at least nineteen sites complementary to 4S RNA in the HeLa mitochondrial genome, twelve

on the H strand and seven on the L strand. These values are in reasonably good agreement with the results of Lynch and Attardi (1976), who found that twelve different aminoacylated tRNAs hybridized to H strand, while five tRNAs bound to L strand. As noted in the Introduction, the number of tRNA genes, as determined by following the hybridization of labeled aminoacylated tRNAs to DNA in excess, may be underestimated if there are multiple identical genes or genes coding for isoaccepting species on the same stand, or if the concentration of one or more aminoacyl-tRNA species are too low either in mass or because of low aminoacylation efficiencies by mitochondrial synthetase in vitro.

The HeLa Mitochondrial 4S RNA Gene Map

The HeLa mitochondrial 4S RNA gene map, illustrated in Figure 5, shows that the nineteen genes are distributed rather widely over the genome, with no apparent simple and regular pattern. More genes are present on H strand than on the L strand. This fairly uniform arrangement of 4S RNA genes is in contrast with the positions of the tRNA genes in yeast mtDNA. In this DNA, eleven of twelve genes mapped so far appear to be clustered in about one third of the genome (Fukuhara et al., 1976). The detailed comparison of the arrangement of individual specific tRNA genes between different mitochondrial genomes may provide interesting information regarding their evolution and function.

The number of mtDNA-specified tRNA species is not known. If the nineteen 4S RNA sites we have mapped actually represent genes coding for mitochondrial tRNA, then the genome has the capacity to code for most, if not all, of the tRNAs necessary for synthesizing proteins in the mitochondrion.

Experimental Procedures

RNA and DNA

Two 4S RNA HeLa samples were prepared from mitochondria as described by Lynch and Attardi (1976). One sample was additionally purified by passage through a column of oligo(dT)-cellulose to remove any small poly(A)-containing RNA fragments.

12S and 16S rRNA were prepared according to the method of Aloni and Attardi (1971b).

For mapping H strands, HeLa mtDNA was prepared according to the method of Aloni and Attardi (1971c). Closed circular DNA was isolated as the lower band after two cycles of CsCl-EthBr equilibrium centrifugation. Relaxed DNA molecules containing on the average a single break per circle were produced by controlled x-ray nicking and isolated as the upper band in CsCl-EthBr gradients (Sharp et al., 1972).

For mapping light strands, pure light strands containing 30% intact circles were prepared as follows by P. J. Flory. To minimize the extent of breakage during strand separation in alkaline CsCl gradients, covalently closed mtDNA labeled in one strand with BrdU was prepared. While unlabeled covalently closed mtDNA is resistant to nicking by alkali, mtDNA strands containing BrdU have an increased sensitivity to such breakage. Thus closed circles labeled with BrdU in one strand will, in alkaline CsCl gradients prepared under appropriate conditions, give rise to largely intact unlabeled strands and highly degraded labeled strands (Flory and Vinograd, 1973; Flory, 1974).

To have a reference point from which to map 4S RNA sites, unique sequence markers were isolated. DNA complementary to 12S and 16S mitochondrial rRNA (rDNA) was prepared by hybridizing denatured mtDNA with rRNA in vast excess. For the hybridization, 10 μg of a preparation of mostly intact ^{3}H-thymidine-labeled H strands (from BrdU-labeled hybrids) were incubated with 40 μg of either 12S RNA or 16S RNA in 2 ml of 0.3 M NaCl, 0.001 M EDTA, 0.01 M Tris (pH 8.0) (25°C) at 60°C for 1 hr. The nucleic acids were collected by ethanol precipitation and centrifugation, dissolved in 1.7 ml of 0.25 M NaCl, 0.03 M sodium acetate (pH 4.6), 5 × 10^{-4} M $ZnCl_2$, and, after addition of 30 μg of denatured HeLa cell DNA, digested with 25 units of a highly purified single-stranded DNA-specific S1 endonuclease from Aspergillus oryzae (a gift from L. Grossman) for 1 hr at 37°C. After addition of EDTA to 0.01 M, the nucleic acids were precipitated with 2 vol of ethanol, dissolved in 2 ml 0.4 M CsCl, 0.001 M EDTA, 0.01 M Tris buffer (pH 8.0), and digested with pancreatic RNAase (10 μg/ml) at room temperature for 30 min to degrade the unreacted RNA. After addition of sodium dodecylsulfate to 0.5% and pronase to 50 μg/ml, the sample was incubated for 45 min at room temperature and then extracted with phenol. Traces of phenol were removed from the aqueous phase by extraction with ether, and the residual ether by bubbling nitrogen. CsCl was added to give a final density of 1.75 g/ml, and the rDNA-rRNA hybrids were centrifuged at 35,000 rpm in a SW 65 Spinco rotor for 5 days at 20°C using a polyallomer tube. The rRNA:DNA hybrids banded sharply at a density of 1.781 g/cc. For comparison, H strand mtDNA banded at a density of of 1.724 g/cc. The fractions corresponding to the hybrid peak were pooled and precipitated with 2 vol of ethanol.

In addition to rDNA, another sequence marker was isolated as one of the fragments obtained by digesting mtDNA with HpA II restriction endonuclease and fractionating the digest by electrophoresis on 2% agarose gels.

Synthesis of N-Hydroxysuccinimide Esters of Biotin, Bromoacetic Acid, and Dithiodiacetic Acid

The synthesis of N-hydroxysuccinimide esters of biotin (NHS-biotin) and of bromoacetic acid (NHS-BrAc) are described by Becker, Wilchek, and Katchalski (1971) and Pellegrini, Cen, and Cantor (1972). The N-hydroxysuccinimide ester of dithiodiacetic acid (NHS-dithiodiacetic acid) was synthesized similarly (N. D. Hershey, personal communication).

Synthesis and Purification of 4S RNA-Biotin Conjugates

Approximately 600 μg of ^{3}H HeLa 4S RNA (4400 cpm/μg), which had been purified through two cycles of sucrose gradient centrifugation (Lynch and Attardi, 1976), were ethanol-precipitated, dissolved in 1 ml of 2 M Tris buffer (pH 8.2), and incubated for 90 min at 37°C to insure that all tRNA molecules were completely deacylated (Sarin and Zamecnik, 1964). The RNA was then oxidized with 0.1 M $NaIO_4$ at pH 4.7. A Schiff base was formed by reaction with diaminopentane at pH 9.3 and stabilized by treatment with $NaBH_4$. The distal amino group of the diaminopentane was acylated with NHS-biotin, and the resulting RNA-biotin conjugate was purified by affinity chromatography on avidin-sepharose.

The affinity column was prepared by incubating 20 ml of CNBr-treated sepharose 4B with 20 mg of avidin for 20 hr at 4°C (pH 7.0) (Bodanszky and Bodanszky, 1970). The modified gel was quenched with 0.04 M aminoethanol for 1 hr at room temperature and extensively washed to remove avidin or avidin subunits not firmly attached. The effective biotin-binding capacity of avidin-sepharose was determined by measuring the amount of ^{14}C-biotin which could be bound in 1 M NaCl and eluted with 6 M guanidine HCl (pH 2.5). In these experiments, two different preparations of avidin-sepharose bound 14 and 5.7 nmoles of biotin per ml of gel, respectively.

The affinity column has several classes of binding sites of varying affinity for biotin (Green and Toms, 1973), and RNA-biotin conjugates cannot be quantitatively recovered from the stronger binding sites. The column was therefore first preloaded with free biotin, and weak sites were liberated by washing the column with 6 M guanidine HCl (pH 2.5). 4S RNA-biotin conjugates were bound to such a preloaded column in high salt [1 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA (pH 6.8)]. After washes with solutions of high salt and high salt containing 2 M urea to eliminate nonspecific ionic or hydrophobic binding, the 4S RNA-biotin conjugates were eluted from the column with 6 M guanidine HCl (pH 2.5), and immediately dialyzed against 1 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA (pH 6.8) for rechromatography on avidin-sepharose. After the first passage through the avidin affinity column, 35% of the material bound; >85% of this fraction bound on a second passage. The 4S RNA-biotin conjugates were stored at −70°C in 1 × SPE [0.1 M NaCl, 0.01 M sodium phosphate (pH 7.0), 0.001 M EDTA]. Details of these procedures will be presented elsewhere (T. R. Broker et al., manuscript in preparation).

Synthesis and Purification of Ferritin-Avidin Conjugates

Ferritin was isolated from horse spleen by procedures similar to those described by Granick (1946). Bromoacetylated ferritin was prepared by reaction of the protein with NHS-BrAc at pH 9.3. In different preparations, between 10 and 20 moles of bromoacetate were bound per mole of ferritin (molecular weight = 9×10^5; $\epsilon^{1\%}_{280\,m\mu} = 14.4$).

Sulfhydryl groups were added to avidin as follows. Avidin was acylated by reaction with NHS-dithiodiacetic acid at pH 9.3. This step was followed by treatment with DTT to expose the SH groups. After removing excess ester and DTT from the reaction mixture by dialysis against 1 × SPE under argon, the number of additional SH groups per avidin was assayed by determining the nondialyzable binding of ^{14}C-N-ethylmaleimide. In different preparations, values between 2 and 6 moles of sulfhydryl groups per mole of avidin (molecular weight = 6.8×10^4; $\epsilon^{1\%}_{280\,m\mu} = 1.54$) were obtained.

Covalent coupling of modified ferritin to modified avidin was performed by mixing ferritin-$(CH_2Br)_{10-20}$ (8.2–10.4 μm) with avidin-$(SH)_{2-6}$ (20–27 μm) in 0.3 M potassium borate buffer (pH 9.3) in the presence of argon. After 2 hr at room temperature, the reaction was quenched by treatment with 0.38 M aminoethanol (pH 9.0) for 1 hr at room temperature. Unreacted avidin was removed by velocity centrifugation of the ferritin-avidin conjugates twice in 5–50% sucrose gradients containing high salt [1 M NaCl, 0.01 M sodium phosphate, 0.001 MEDTA (pH 6.8)]. After sedimentation for 4 hr at 40,000 rpm in an SW 50.1 rotor at 10°C, ferritin-avidin bands about ⅓ of the way down the tube. For these electron microscope labeling experiments, preparations of ferritin-avidin capable of binding 2.5–4.4 moles of biotin per mole ferritin were used. Ferritin-avidin was stored in 1 × SPE at 2°C. Additional information concerning the synthesis of ferritin-avidin conjugates will be reported elsewhere (T. R. Broker et al., manuscript in preparation).

Hybridization Procedures

Light Strand Mapping

Pure L strands prepared as described above were hybridized to marker DNA sequences, that is, either rDNA or HpA II fragment 3, and to biotin-4S RNA according to the following typical procedure. To insure that no single-stranded H strands were present as contaminants, L strand DNA samples were first incubated under annealing conditions [40% formamide, 0.2 M sodium phosphate (pH 7.5) in early experiments; 40% formamide, 0.3 M NaCl, 0.1 M Tris, 0.001 M EDTA (pH 8.0) in later experiments] to a Cot of 4×10^{-4} mole sec l^{-1} which we estimate to be equivalent to 10 times the Cot$_{\frac{1}{2}}$ for HeLa mtDNA. The 12S and 16S rRNA-DNA hybrids were denatured by treatment with 0.1 M NaOH for 4 min at 50°C and titrated with Tris-HCl to pH 9.5. HpA II fragment 3 duplexes were denatured by heating to 90°C for 5 min in 0.002 M Tris, 0.002 M EDTA (pH 7.4). These single-stranded marker DNA sequences (either 12S and 16S rDNA or HpA II fragment 3 DNA) and biotin-4S RNA were added to self-annealed L strands, dialyzed against the annealing buffers described above, and incubated for 3.5–4 hr at 40°C. In these incubation mixtures, the L strand DNA was at 1 μg/ml; the 12S + 16S rDNA at 1–1.5 μg/ml; the HpA II fragment 3 at 0.8 μg/ml; and the biotin-4S RNA at 20–30 μg/ml. These conditions are approximately equivalent to a C$_0$t of 70×10^{-3} for 4S RNA-biotin. O. Uhlenbeck and his co-workers (personal communication) have determined that the C$_0$t$_{\frac{1}{2}}$ for 5S RNA in 50% formamide, 2 × SSC, 40°C is $3–4 \times 10^{-4}$. We predict that the C$_0$t$_{\frac{1}{2}}$ for tRNA under the same conditions would be 3×10^{-4}; for 20 different species present in equal concentration, the C$_0$t$_{\frac{1}{2}}$ would be approximately 6×10^{-3}.

Heavy Strand Mapping

Because isolated mtDNA H strands were not available, these mapping experiments were carried out with singly nicked total mtDNA. H strands were distinguished from L strands by the presence of duplex regions after hybridization with HeLa mitochondrial 12S and 16S rRNA. To minimize the problemm of DNA-DNA reassociation during the hybridization, more stringent annealing conditions in a high formamide solvent were used, thus favoring the formation of RNA:DNA hybrids over DNA:DNA duplexes. (R. White and J. Casey, personal communications.) Singly nicked mtDNA (0.1 μg) was denatured in 0.1 M NaOH for 3 min at room temperature, and then the pH was adjusted to 8.5 with Tris-HCl. Ribosomal RNA and 4S RNA-biotin were added, and the mixture was dialyzed at 4°C for 3 hr against annealing buffer [80% formamide, 5 × SSC, 0.03 M Tris (pH 7.5)]. After dialysis, the volume of the reaction mixture was adjusted to 0.2 ml to give a DNA concentration of 0.5 μg/ml; a 12S rRNA concentration of approximately 6 μg/ml; a 16S rRNA concentration of approximately 12 μg/ml; and a 4S RNA-biotin concentration of 23 μg/ml. The hybridization mixture was incubated for 35 min at 46°C. With respect to 4S RNA-biotin, the conditions correspond to an approximate C$_0$t of 15×10^{-3} moles sec l^{-1} (estimated C$_0$t$_{\frac{1}{2}} = 6 \times 10^{-3}$).

Labeling of 4S RNA-Biotin-mtDNA Hybrids with Ferritin-Avidin

After the hybridizations were completed, excess 4S RNA-biotin and ribosomal gene region markers were removed by passing the reaction mixtures over a 3 ml column of Sepharose 2B equilibrated with 0.1 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA (pH 6.8). The excluded volume was collected and concentrated under vacuum 5–7 fold to 50–75 μl. An equal volume of ferritin-avidin solution was added to give a final ferritin concentration ranging from 0.45 to 1 mg/ml. After incubation for 16 hr at room temperature, the mixture was layered on a solution of sodium lothalamate (ρ = 1.4), buffered with 0.1 M Tris, 0.001 M EDTA (pH 8.5), and centrifuged for at least 10 hr at 35,000 rpm at 15°C in an SW 50.1 rotor. Since the density of DNA in sodium lothalamate is 1.14 (Serwer, 1975), while that of ferritin is estimated to be 1.6–1.8, ferritin-labeled hybrids can be separated from excess ferritin-avidin by this method. The DNA-containing fractions (0.2–0.6 ml from the top of the gradient) were collected manually from the top and dialyzed first against 0.8 M NaCl, 0.1 M Tris, 0.01 M EDTA (pH 8.5), and then against 0.2 M Tris, 0.02 M EDTA (pH 8.5). The samples were concentrated 3–4 fold in a vacuum desiccator and redialyzed versus 0.2 M Tris, 0.02 M EDTA (pH 8.5) in preparation for spreading for electron microscopy.

Electron Microscopy

The electron microscopic procedures used here are described in more detail by Davis, Simon, and Davidson (1971). The spreading solution contained 50% formamide, 0.1 M Tris, 0.01 M EDTA (pH 8.5), and 50 μg cytochrome c/ml. Depending upon the fraction taken from the lothalamate gradient, the DNA concentration was 0.01–0.05 μg/ml. The hypophase consisted of 17% formamide, 0.01 M Tris, 0.001 M EDTA (pH 8.5). The DNA was picked up on parlodion-coated grids, stained with 10^{-4} M uranyl acetate, and shadowed with 3–3.5 cm of platinum-palladium wire at a shadowing angle of 9:1.